University of Alberta

MHC Regulation in Non-Lymphoid Tissues in the Mouse

by

Tasha Nicholle Sims ©

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ABSTRACT

Major histocompatibility complex (MHC) molecules present antigen to T cells and are critical for T cell development and activation in host defence against infection, autoimmune disease and graft rejection. Little is known about regulation of MHC in non-lymphoid tissues. Since kidney is a transplantable organ it is important to understand MHC-dependent recognition of kidney by the host. I sought to understand mechanisms of MHC expression in kidney.

This lab defined several states of MHC induction in kidney: the basal state, the response to inflammatory stimuli, and the response to injury and transplantation. I found that MHC class I expression in the basal state was partially dependent on IFN-γ. In response to inflammation, MHC expression was completely dependent on IFN-γ. The antigen presenting machinery genes are also responsive to IFN-γ and IFN-γ-inducing stimuli. Thus the antigen presentation response to IFN-γ in the kidney is not simply from the MHC molecules *per se*.

The MHC class II transactivator (CIITA) is a regulator of class II expression. I cloned and sequenced mouse CIITA. In CIITA gene-disrupted mice, all inducible class II expression in the kidney was dependent on CIITA. Even in responses such as inflammation, ischemic injury or graft rejection, class II was completely CIITA-dependent. The absence of CIITA did not result in a decrease in class I. This is an important finding since it was proposed that class I was dependent on CIITA.

I found that the promoters of CIITA were differentially regulated in kidney in response to various stimuli. The IFN-γ sensitive promoter use reflected total CIITA mRNA induction in inflammatory states. All promoters were used in kidney in response to ischemic renal injury, reflecting the complexity of the injury response. To our knowledge these are the first studies of CIITA promoter usage *in vivo*.

Thus I have demonstrated multiple levels of MHC regulation in kidney. These studies contribute to the understanding of MHC regulation in tissue. I show that the mechanisms sometimes differ *in vivo* from what was previously thought based on *in vitro* data, an important consideration if we are to understand the mechanisms of graft rejection or acceptance.

"The most beautiful thing we can experience is the mysterious; it is the source of all true art and science."

Albert Einstein

Dedicated to:

Edward Ernest Farr

1915-1999

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ABBREVIATIONS

aa	amino acid
APC	antigen presenting cell
ATN	acute tubular necrosis
CIITA	MHC class II transactivator
CIITAKO	129xB6 mice with disrupted CIITA genes
CRE	Class I Regulatory Element
CRC	Class I Regulatory Complex
GAS	Interferon γ-activated site
GKO	BALB/c mice with disrupted Interferon-γ genes
GRKO	129/J mice with disrupted Interferon-gamma receptor α chain genes
HPRT	Hypoxanthine Phosphoribosyltransferase
ICS	Interferon Consensus Sequence
IFN-γ	Interferon-gamma
IIP	indirect immunoperoxidase
i.p.	intraperitoneal
IRE	Interferon response element
ISRE	Interferon-stimulated response element
IRF-1	Interferon regulatory Factor 1
IRF-1 KO	129/J mice with disrupted Interferon regulatory Factor 1 genes
ISRE	Interferon stimulated response element

KO	"knockout", gene-disrupted mouse
LMP	Low molecular weight polypeptide
LPS	Lipopolysaccharide from *Salmonella minnesota*
MHC	Major histocompatibility complex
PI	CIITA promoter I
PIII	CIITA promoter III
PIV	CIITA promoter IV
P815	mastocytoma tumour from a DBA/2 mouse background
RABA	radio labelled monoclonal antibody binding assay
rIFN-γ	recombinant interferon-γ
RABA	radiolabelled monoclonal antibody binding assay (to tissue homogenates)
RFX	regulatory factor X
RT-PCR	Reverse transcriptase -polymerase chain reaction
STAT	signal transducers and activators of transcription
TAP	Transporter associated with antigen presentation
TCR	T-cell receptor
WT	Wild-type mouse
5'-UTR	5 prime untranslated region

Chapter 1

Background

I. INTRODUCTION

During an acquired immune response, T cell activation is strictly dependent on expression of major histocompatibility complex (MHC) products and the display of antigens within MHC molecules. MHC product expression is relevant to naive T cell development and T cell activation in host defence against infection, in graft rejection and in autoimmune disease. In addition, peripheral CD8 and CD4 T cells require stimulation through their antigen receptors to survive (1-4), indicating a role for both MHC class I and II product *in vivo* in maintaining the peripheral T cell population (2;3). The role for MHC in maintaining memory cell populations, however, is minimal (5;6).

MHC genes (H-2 in mouse and HLA in human) are expressed or inducible in many tissues. MHC product is expressed at a basal level in the healthy host and is increased during infections, tissue injury and transplant rejection, and autoimmune disease (7-13). The basal level of MHC expression in each cell reflects a variety of influences including constitutive expression and induction by exogenous signals from cytokines (i.e. interferon-gamma, IFN-γ), extracellular matrix, effects of tissue injury and repair (14;15), and changing cell populations.

Therefor, patterns and levels of MHC expression in tissues will be related to the T cell response in the context of each tissue.

II. HISTORY

The study of transplant immunology led to the discovery of the MHC genes. In the 1800's, mouse fanciers had created lines of inbred mice that had predictable coat colour or behaviours. In 1901, Loeb and colleagues became interested in tumours and used the inbred strains to carry and passage the tumours (16). Between 1905 and 1912, Ehrlich and Bittner described sensitisation and tolerance when they discovered that some mice succumbed to the tumours while others overcame them. The basis for this survival was unknown but it was seen that the (AxB) F1 generation would accept a tumour of either A or B type (where A and B are inbred lines). From 1909-16 Little and Tyzzer published landmark papers describing the mathematical relationships among the generations and the tumours they would accept (17;18). These were the first transplant genetic relationships described. These factors were genes encoding antigens that rendered the host intolerant to the tumour graft. These finding were the basis of the three laws of transplantation: 1) isografts accepts, 2) allografts fail, and 3) acceptance is governed by multiple co-dominant Mendelian factors.

These basic observations set the stage for a debate about transplantation that is still unresolved: the issue of the humoral immunity or cellular immunity mediating graft rejection. In 1936 Peter Gorer showed there were separate blood

groups in serum and that histocompatibility antigen 2 (H2) was associated with strong tumour rejection (19). Landsteiner and Chase described delayed-type hypersensitivity in 1942 and showed that both delayed type and contact hypersensitivity reactions are mediated by cells (20). The histocompatibility gene was discovered to be a complex in 1951, when George Snell discovered the K and D loci of H2 (21). Human MHC was then described by Dausset, Payne and van Rood. The discovery of the MHC won the Nobel Prize in 1980, awarded to Dausset, Benacerraf and Snell. In 1953, Hasek and Medawar, Billingham and Brent found that skin grafts on mice behaved within the parameters of an immune response; their work was the foundation for the understanding of rejection and tolerance (22). Immunologic tolerance won the Nobel Prize in 1960, which was awarded to Medawar and Burnet. In 1964, Barbara Bain in Montreal was the first to describe that the mixed lymphocyte reaction resulted in the proliferation of cells (23). Although work focussed on cell-mediated rejection for decades, recently the importance of antibody in transplant immunology has re-emerged.

MHC class II was described using techniques founded with the proponents of cell-mediated immunity. The mixed lymphocyte reaction between two "identical" K/D recombinants was found to yield proliferation, indicating there must be other determinants in the MHC. In 1963, Benacerraf discovered that in rabbits there was some genetic mechanism controlling the immune response to copolymer antigens (24). Hugh McDevitt, using purified antigen described that the MHC, and specifically the "I" region, controlled the immune response. The

work by Zinkernagel and Doherty in 1974 won the Nobel prize for MHC restriction, linking MHC and T cell recognition together in immunity (25).

One of the most important recent contributions to the study of the MHC has been the crystal structures of the molecules along with the sequencing of the MHC region. Pam Bjorkman first crystallised class I in 1987 defining for immunologists the molecule responsible for the phenomena described over many decades (26;27). Wiley's group followed with the crystal structure of class II in 1993 (28). In 1996, class I, peptide and the TCR together were finally visualised through their co-crystal structure (29;30). In 1999, the full sequences of human and chicken MHC were elucidated (31;32).

III. TRANSPLANTATION AND TISSUE INJURY

Transplantation is the act of transferring cells from one site to another. In the case of allotransplantation, organs (usually) are transferred from one non-identical individual to another. This treatment is effective in diseases involving the kidney, liver, lung, heart, cornea, skin, islets etc. In the present era, with the advent of immunosuppressive drugs such as cyclosporine, the procedure has a high probability of success. Challenges remain since the mechanisms of graft rejection are not resolved.

MHC molecule expression is induced in response to allogeneic stimulation, and this induction is relevant to T cell activation in graft rejection. In the United States, cadaveric kidneys transplanted to human leukocyte antigen

(HLA)-matched recipients were projected to function twice as long as kidneys with HLA mismatches (33). Cytokines mediate MHC expression but ischemic-reperfusion injury and other types of non-specific injury also induce MHC molecules. Injury induces a complex pattern of gene expression and affects various molecules with important roles in the immune response (34-37). Ischemic reperfusion injury is a pathophysiologic response comprised of a complex and robust set of mechanisms including leukocyte rolling and extravasation, indicating interactions with the endothelium. Ischemic reperfusion injury to allografts could affect immune reactions against that tissue since inflammation and the induction of immune mediators are hallmarks of injury (38). The antigen-presenting cell may initiate injury or rejection through reactive oxygen radicals, upregulation of MHC products, and increases in adhesion molecule expression that can then affect T cell recognition. It is likely that both class I and class II antigen presentation systems play an important role in tissue immunogenicity in transplantation (39). The effect of injury on tissue immunogenicity could thus play a role in the increased rejection and graft loss from cadaver kidney transplantation versus living donor transplantation (40).

A. Transplantation

MHC alloantigens appear to be the major target of the rejection response. During graft rejection, class I and II molecules are massively induced both in the rejecting organ and in the host (8). The contributions of cytotoxic T cells, T

helper cells or alloantibody are not entirely clear, all likely acting in concert in the normal situation to reject a graft.

The first set of rejection responses is characterised by infiltration of host immune cells (8). The cellular infiltrate of a graft contains cytokine-secreting cells such as macrophages and lymphocytes. In addition to cytokines, macrophages secrete proinflammatory factors such as growth factors, enzymes, and procoagulant activities that may contribute to cell injury and dysfunction in vascularised grafts undergoing a rejection episode. Candidate effector mechanisms leading to graft rejection include delayed type hypersensitivity, cytotoxic cells, or antibody. Damage to the graft by antibody-dependent mechanisms include complement deposition, antibody dependent cellular cytotoxicity, or Fc receptor mediated activation of macrophages. Direct lysis by natural killer or cytotoxic T cells could damage the graft through cellular means. The relative importance of these mechanisms for graft rejection is unknown.

Rejection episodes fall into three categories: 1) Hyperacute rejection, 2) Acute rejection, or 3) Chronic allograft dysfunction. Hyperacute rejection is mediated by preformed antibodies to HLA, ABO blood groups or endothelium (41-43). Hyperacute rejection is demonstrated within minutes or hours due to the fixation of complement by preformed antibodies deposited on the endothelium. Acute rejection begins by day 5 to 7 after transplantation and is marked by inflammation and specific cell injury and cell death. The inflammation is manifest by infiltrating leukocytes, accompanied by edema and reduced blood flow (44). This reduced perfusion, along with damage caused by infiltrating cells, causes

loss of function. Further, early success with a transplant does not ensure good graft function later on (45). In kidney, the cellular stresses on an older, overworked transplant from a suboptimal donor contribute to chronic allograft dysfunction (46-48). This stage of rejection is characterised by intimal thickening, interstitial fibrosis and tubular atrophy.

The role of IFN-γ in the rejection of allografts is unclear. One would hypothesise that IFN-γ is a destructive mediator of graft rejection since it is a proinflammatory cytokine. Wild-type mice have increases in class I and II molecule levels both in the graft and in the host during allograft rejection and there is massive induction of IFN-γ. Class I and II are strongly upregulated on parenchymal and endothelial cells in the graft (unpublished data, Afrouzian, M. and P.F. Halloran). The long-term effects of IFN-γ may render the graft immunogenic to the host. The early effects of IFN-γ on chemokines, nitric oxide production, or cytokine profiles during graft rejection have not been addressed.

The expression of CD40 on the antigen presenting cells (APC) is the determining factor in T cell help for CD8 cells (49-51). APC express both MHC class I and II (discussed later), making the APC a target for investigating immunity and transplantation. Two models of antigen recognition are proposed for graft rejection: direct and indirect presentation (52). Direct recognition of foreign MHC class II by CD4 T cells involves host T cells responding to donor MHC on donor antigen presenting cells. APC in this case include resident dendritic cells (DC) such as the Langerhan cells of the skin or the Kupffer cells in the liver or other resident cells such as endothelial or epithelial cells. These cells

vary both in numbers and in their function as efficient APC. Indirect recognition of foreign class II by host T cells, on the other hand, involves the host cells presenting peptides derived from donor MHC in the groove of host MHC. Although the indirect pathway is closer to the normal immune response (i.e. self T cells react to self MHC with a foreign peptide), it is considered to be less important in the context of graft rejection (53), although this view is being revisited. Peptides presented by the indirect pathway include MHC peptides as well as minor peptides. Here, the antigen presenting cells are likely mature DC in the draining lymph node.

The role for MHC class I in allograft rejection is not clear. In humans, most HLA matching is done on the basis of class I, with only DR matched for class II. Long-term survival of β2 microglobulin gene-disrupted (β2KO) pancreatic islet allografts in wild-type mice indicates that class I in the graft is important in rejection (54). However, in a heterotopic vascularised cardiac model where the donor mouse heart is transplanted into the neck of the recipient, class I deficiency was shown to only slightly increase the survival time of the graft and a similar amount of infiltrate was seen as in the wild-type allograft controls (9). Further, data from a β2KO orhtotopic kidney allograft model (where the native kidneys are removed at the time of surgery and at day four) indicate that reduced direct presentation is not sufficient to protect the kidney from chronic rejection by a wild type mouse at six weeks (55). However, at four weeks, the lack of class I on the kidney protected the function of the kidney as measured by inulin clearance, although histologically the chronic rejection process was proceeding

normally (56). The differences in these studies may be due to the models. Islet and skin grafts are often revascularised by host circulation, making the graft a chimera with host tissue and thereby less likely to be rejected than other whole organ vascularized transplants.

Looking at class II, skin graft rejection occurs across class II differences between H-2^b and the class II mutation bm12 (57). Data from skin grafts and tumour grafts in class II KO mice show that the indirect pathway is sufficient to mediate graft rejection. Furthermore, in a class II deficient hetertopic cardiac allografts (as above) model, long-term survival occurred after a bout of initial swelling and decreased function (9;53). However, class II KO mice (H-2^b, I-A$\beta^{-/-}$) express the I-Aα chain and the entire class II presenting machinery, but display no stable α/β heterodimers on the cell surface. Because the class II machinery is still available for peptide generation there remains doubt as to the validity of the experiments in class II KO mice with respect to determining if the indirect vs. the direct pathway are the most important for graft rejection.

Further evidence for the importance of indirect presentaion of MHC molecules in allorecognition came from studies with denatured class I and II heavy chains in an immunisation protocol. The immunised denatured proteins stimulated an antibody response to themselves and to whole MHC molecules and decreased the skin graft survival time in a rat model (58). Thus, although the MHC molecule is not presented on the cell surface in the class I deficient (β2KO) and class II KO mice, the heavy chains are still available as peptides to stimulate the immune response. Although it is accepted that MHC molecules play

a role in rejection, especially acute rejection episodes, the exact roles of class I or II in graft recognition are unclear (36;48;59-61).

B. The Injury Response

Injury to a tissue is thought to be a leading cause of graft rejection and may play a role in transplant loss by increasing the predisposition towards immune recognition and rejection (62). Delayed graft function is a clinically important form of renal injury (acute tubular necrosis, ATN) and increases the risk of graft loss both early and late in the life of the graft. If there is no rejection during the recovery period after ATN, then the risk of graft loss decreases (45). Thus ATN seems to be a predisposing factor to rejection, but it can be overcome. However, ATN also affects late graft loss, and this likely reflects the risk of early acute rejection episodes, likely mediated by MHC among other factors. Of interest, living unrelated donors have good graft survival despite MHC mismatching, indicating the importance of non-MHC mechanisms. This high rate of graft survival may be due to the fact that there is no brain death or storage of the organ and thus less injury (36). The mechanisms through which injury mediates downstream events are poorly defined, however.

Injury induces MHC molecules. There are several experimental systems for inducing injury in the kidney. In addition to ischemia-reperfusion injury, several agents are nephrotoxic. These agents include the antibiotic gentamicin, mercuric chloride, the immunosuppressant cyclosporine, and the

chemotheraputic drug cisplatin. The response to injury is local, stereotyped, and independent of the injurious agent (ischemia versus toxins). In a unilateral ischemic ATN (acute tubular necrosis) mouse model, the contralateral kidney, serving as a control, shows neither injury nor enhanced MHC molecule production. Acute kidney injury alters the expression of genes in renal epithelium and within the interstitial cell population. Beginning several days after renal injury, likely during the regeneration phase, class I and class II expression are transiently induced in epithelium of the injured kidney (63). This induction is weaker (about a 2-3-fold increase) than observed with systemic stimuli (about a 10-fold increase) and is accompanied by an increase in class II positive interstitial cells (14;64-66). The mechanisms of MHC molecule induction in epithelium are not known. But the changes in MHC molecule levels may be relevant to the ability of injury to alter the immunogenicity of the tissue in autoimmune disease and transplantation (37)}

In addition to MHC molecule induction, heat shock protein-72, chemokines, cytokines, adhesion molecules, nitric oxide and growth factors are also induced in response to renal injury (34;67-69). Many of these responses can be attributed to the cytokines IFN-γ, TNF-α, IL-1 that are released in response to renal ischemic injury (69-71). In hearts, ischemic injury has been associated with the activation of the MAP kinase pathway (72), which may also contribute to the induction of several factors involved in the injury response.

IV. REGULATION OF THE MAJOR HISTOCOMPATIBILITY COMPLEX

T cells recognise antigen only in the context of MHC. In very general terms, antigen is separated into two pools to be presented by MHC molecules. Intracellular antigen is presented by class I to CD8 T cells while extracellular antigen is presented by class II to CD4 T cells (Figure 1.1). Although there are many examples of cross-priming between the two systems, the relevance or mechanism behind the alternate presentation pathways is unclear (73) and will not be discussed further here. In order to stimulate different immune responses, the types of antigens presented by class I and class II differ. Table 1.1 outlines the variations in the antigen pools and molecules involved in antigen presentation.

MHC molecules in human and mouse share similar regulatory features and are expressed under similar circumstances. Class I molecules consist of a heavy chain of which there are three varieties. In human they are called HLA-A,B or C while in mice they are called H-2K,D, or L. Class II molecules on the other hand are made of two chains, α and β and both must be available to see cell-surface expression. In human the class II molecules are called HLA-DP, DQ or DR and in mice they are called I-A or I-E. This discussion will focus on the regulation of MHC expression, specifically class I, class II and the class II transactivator CIITA.

A. MHC class I

Antigen presentation to a CD8 T cell requires the MHC class I molecule to be loaded with peptide, usually derived from the cytosol or endoplasmic reticulum (ER) (Figure 1.2). Most nucleated cells express class I in the basal state and inflammatory stimuli can upregulate class I on those cells. The major molecules that will be discussed in this section are involved in the pathway to present mature class I:peptide on the cell surface. Mature class I complexes are non-covalently associated trimers of a 45kDa heavy chain, β2-microglobulin (β2m), and a peptide. The molecules involved in the pathway include cytosolic proteasomal subunits, the peptide transporters located in the ER membrane, and various chaperones.

Cytosolic proteins are processed by the ATP-dependent 26S large molecular weight proteasome that degrades both ubiquitinated and non-ubiquitinated proteins. The proteasome is conserved throughout evolution and is constitutively expressed in all tissues (74). It is thought that the proteasome is the major source of peptides for class I presentation although inhibition of proteasome function does not halt all class I epitope generation, suggesting other proteases may be involved (75-77). The roles of other proteases remain unclear and may only be evident when the proteasome is non-functional (78). The inhibition of proteasomal function and subsequent induction by IFN-γ suggest that proteasome function is a limiting step in class I presentation (79);(80). Proteins undergo partial degradation by the proteasome into peptides that are transported

to the ER via the transporter for antigen presentation (TAP), which is discussed below.

Evidence for the importance of the immunoproteasome comes from studies in the PA28 gene-disrupted (PA28KO) mice, which have defects in CTL responses (81). IFN-γ-induced PA28α/β heterodimers activate the 20S proteasomes and promote the assembly of the "immunoproteasome" and the ubiquitin-independent double cleavages of substrates (82;83). Unlike wild-type mice, PA28KO mice have almost no low molecular weight protein (LMP) 2/7/10 subunits in their proteasomes after IFN-γ stimulation and have impaired class I antigenic epitope repertoires.

The incorporation of several subunits into the proteasome may increase class I epitope generation. LMP 2, 7 and 10 (MECL in human) are IFN-γ-inducible catalytic subunits of the vertebrate 20S cytosolic proteasome complex that displace three constitutively active subunits (δ, X and Z, respectively) (84-86). The co-operative incorporation of the inducible subunits in order (LMP 7, 2 then 10) alter the immunoproteasome specificity and enhance the generation of peptides of 8-10 amino acids (aa) with hydrophobic ends, which is consistent with class I molecule-binding peptides (87;88). Cells deficient in LMP2 and 7 show altered catalytic activities compared to normal cells and LMP+ proteasomes cleave rapidly after basic and hydrophobic residues (89;90). A role for LMP2 and 7 has been linked with the presentation of specific antigens, such as the influenza A antigen and the HY antigen, respectively (91-93). Both genetic and biochemical evidence suggest that LMP7 is fundamental for induced antigen

presentation (91;74). LMP2 gene-disrupted mice have fewer mature CD8 cytotoxic T lymphocytes compared to wild-type mice and LMP7 gene-disrupted mice have few cell surface class I molecules (74). This indicates the importance of stable class I molecules in the maturation and maintenance of the peripheral CD8 T cell population and the contribution of the immunoproteasome subunits to generating epitope diversity for class I (91;94).

From the cytosol, peptide entrance to the ER is regulated. TAP 1 and 2 form a multispan heterodimer located in the ER membrane, are ATP-dependent, and are members of the ABC family of transporters (i.e. drug resistance channel, MDR and the chloride channel, CFTR) (95). Transport of peptides requires both TAP subunits and ATP for peptide transport (not for peptide binding) and prefers peptides between 7 and 12 aa. TAPs are encoded in the class II region of the *MHC* and highly conserved in mouse and human. TAP mediates and may selectively transport cytosolic peptides across the ER membrane to bind class I (96-101). In TAP-deficient cells, class I is thermolabile and most molecules are retained in the ER in association with calnexin (101). TAP1KO mice or people with no TAP2 (a rare immunodeficiency) have few class I molecules on the cell surface (102;103) and reduced CD8 CTL counts. HLA-A2 molecules, however, are less dependent on TAP for epitope generation since they are largely loaded with signal peptides from within the ER (104). Taken together the data indicate that peptide availability may be a limiting step in class I antigen presentation, and that TAP may be partially responsible for the repertoire of peptides presented.

Numerous chaperones may trim peptides in the ER and then help TAP load the peptides onto the class I heavy chain:β2m complex (100).

ER-resident chaperones are important to maintain class I stability while it awaits peptide loading. Class I heavy chains and β2m are synthesised and assembled in the ER. The mechanism is not fully elucidated, but it seems that the class I heavy chain associates sequentially with the chaperones BiP (immunologlobulin binding protein) then calnexin to facilitate interactions with β2m (at least in human) (101). Class I binding of β2m leads to a switch in chaperones from calnexin to calreticulin, which is homologous to calnexin and binds Ca^{2+}. Class I:β2m:calreticulin can then complex with TAP1, likely through tapasin (gp48, an MHC encoded, proline-rich Ig super family member and ER resident) (99). Tapasin co-precipitates with TAP and can be found in a 4:1 ratio with TAP. Without tapasin there is a failure to load class I with peptide and class I is subsequently kept in the ER (101). Cim 1 and 2 are also newly-described members of the class I pathway, but their roles remain unclear (105).

In the normal sequence of events class I:β2m:chaperone contacts the TAP:peptide:chaperone complex and peptide is loaded into the class I groove. Many heavy chains are degraded in the ER before interacting with peptide, indicating that peptides or peptide-dependent mechanisms are needed to stabilise the molecules. The cell-surface expression of the class I:β2m:peptide trimer is not only dependent upon expression of the heavy chain but also upon the availability and association of the peptides with nascent class I molecules in

the ER. Once class I is loaded, chaperones disassociate and the stable class I trimer (heavy chain: β2m:peptide) traffics to the cell surface.

The expression of the class I heavy chain is largely regulated at the level of transcription (Figure 1.3, 1.4), although additional mechanisms have been proposed. Theories implicating proteolytic cleavage of the heavy chain and "stealing" of the MHC by T cells are currently being investigated (106;107). The promoters of the class I family of genes are highly conserved among alleles such as H-2K/D/L and in mammals such as rabbit, pig, mouse and human (108). Among other class I promoter-binding transcription factors such as NF-κB, IFN-γ regulates class I through IRF-1binding the ISRE. Some data also implicate the class II transactivator (CIITA) in class I regulation, as discussed below. Heavy chain stability is highly dependent on β2-m, likely due to the multiple interaction points between the two molecules (26) and peptide, as mentioned above.

B. MHC class II

Class II molecules are expressed in the basal state on B cells and dendritic cells, which can then increase class II levels upon stimulation (i.e. inflammation). Endothelial and epithelial cells, usually class II negative, can be induced by IFN-γ to express class II (14). The display of class II molecules on the cell surface is stabilised by the association of peptide within the class II groove (Figure 1.5). Like the class I pathway, the class II pathway has many regulatory players including the invariant chain (Ii) which guides class II to the endosomal

compartment, molecules involved in degradation of Ii, and peptide loading molecules.

The invariant chain is central to trafficking class II to the endosomal pathway to avail class II for binding exogenous peptides. The Ii is non-polymorphic but the name is a misnomer since Ii is expressed as two splice variants. The Ii trimer associates with three class II heterodimers and protects them from being loaded by peptides derived in the ER (109). Ii aids in the proper folding and assembly of class II in the ER by dissociating class II from chaperones such as BiP. Functional regions of Ii have been identified using deletion mapping: the carboxy-terminal and transmembrane regions are used in trimerisation while class II binding depends on aa 81-104 (CLIP) and aa 118-128 (110). The Ii targets the class II molecule toward the endosomal compartments using sequences in the Ii cytoplasmic tail (111). In the endosomal compartment, Ii is degraded through sequential proteolysis leading to the final class II-associated Ii peptide, CLIP. The primary candidate for this degradation is cathepsin S since it is highly expressed in antigen presenting cells and is active at the pH of the endocytic pathway (pH 5-8) (112). In the final stages, CLIP is released and replaced by antigenic peptide to generate class II/peptide complexes. CLIP may render class II receptive to peptide loading since class II preloaded with CLIP is a better substrate for peptide binding than empty class II (113).

Genetic evidence suggests that the introduction of peptide to MHC molecules is a regulated step. The Ii gene-disrupted mice show reduced levels of

class II on the cell surface and a reduced CD4 T cell repertoire (114). The class II that does reach the cell surface in those mice has a more "open" conformation suggesting they are empty or only loosely loaded with peptide. Peptide loading is also regulated at several steps. The IFN-γ independent molecule, HLA-DO (H-2O in mice), regulates peptide availability through HLA-DM (DM), the peptide loader *in vitro*. DM is a heterodimer with approximately 20-fold lower expression than class II. DM is not cell-surface expressed but is found in the late endosomal compartment where it traffics via a YTPL sequence in the β chain cytoplasmic region. HLA-DMA and –DMB (H-2Mα and Mβ1/2), which map to the class II region of the *MHC,* are non-classical MHC molecules with limited polymorphisms. DM molecules have a structure similar to conventional class II but with a closed groove (115;116). Mice deficient in H-2M display a single peptide, CLIP, and the T cell repertoire is severely limited (117-119). H-2M is required for class II-restricted antigen expression on the cell surface and thus is required for the creation and maintenance of the CD4 T cell repertoire.

DM catalyses the removal of CLIP and subsequently facilitates the loading and editing of antigenic peptides onto class II (120; 121; 122). DM likely functions somewhat as a chaperone, stabilising class II in a peptide-exchange prone conformation and changing the kinetics of association while not changing the affinity of peptide association (120;123;124). DM is also thought to be a peptide editor, favouring associations with slow dissociation rates from class II. *In vitro*, DM can bind to and stabilise empty class II and increase the likelihood that peptide will associate with the groove (125). Several rounds of editing may

be required to generate stable class II:peptide trimers that are released to the cell surface.

While there are many regulated steps before class II can associate with peptide and present it to CD4 T cells, class II heavy chain availability is regulated mainly at the level of transcription (126;127). Since the class II family of genes (α, β, Ii, DM) have highly homologous promoter regions and thereby bind the same families of transcription factors (Figure 1.3) (128), co-ordinate regulation of the antigen presentation system is an important feature of class II expression.

Transcriptional regulation of class II

Several transcription factors are able to regulate the class II family due to conserved sequences within the promoter regions of each gene. People deficient in one of these factors have a disorder known as bare lymphocyte syndrome (BLS), where class II is not expressed on their lymphocytes. Patients with BLS have been divided into four groups based on the genetic etiology in each case. Genetic and biochemical evidence has demonstrated that the BLS groups A,B,C, and D are deficient in CIITA, RFX-B/Ank, RFX-5 and RFX-AP, respectively (129-132). The RFX family of transcription factors bind the X-box of the promoters of the class II family (Figure 1.6) and the RFX complex is required for class II transcription. RFX-5 interacts with only the 5' side of the X-box while RFX-B/Ank binds only the 3' side. RFX-AP binds many base pairs throughout the X box. None of the cloned factors alone bind DNA; they are only found as a complex on DNA. Based on these observations, it seems likely that all three

factors bind DNA and that there is a requirement for some interaction among the proteins, perhaps for stability (133). The X2 box is bound by X2BP, likely as a homodimer, and co-operatively interacts with RFX5 (133). X2BP not only interacts with RFX-5 but also with the heterotrimer NF-Y, which binds to the Y-box (134). Chromatin structure is also regulated in class II expression (135) but the molecules involved have not been defined. The transcription factors for the class II family vary among cell types but all are ubiquitously expressed. There remains the question of how class II is so precisely regulated among cell types and in the context of a variety of stimuli .

C. Class II Transactivator (CIITA): Master Transcriptional Regulator of Class II Family

CIITA is essential (136-138) and qualitatively important for most class II expression in numerous cell types and tissues (64;136;139-142). CIITA is sufficient and necessary for basal and induced MHC class II expression (64;137;143). Further, CIITA mRNA expression correlates strongly with class II expression in tissues (64). Because of its relationship to class II, the level of expression of CIITA is relevant to immune maintenance and the immune response in each tissue *in vivo*. CIITA may play a quantitative role in class II and other target gene expression and thus in T cell recognition, since the quantity of class II expressed is critical in T cell recognition (144-147). CIITA deficiency causes a severe immunodeficiency due to failure of CD4 T cell development and activation (129;137). In addition, recent studies in mice with disrupted CIITA

genes have shown a severe generalised defect in all class II expression, with the exception of the thymus (137;148;149), indicating that CIITA is essential for the regulation of class II in the normal immune response. Plasma cells that are developmentally class II deficient have been shown to have silenced CIITA (150;151) and trophoblast cells also fail to express CIITA (152). CIITA does not bind DNA but rather likely functions through the transactivation of a conserved set of DNA binding proteins (the X and Y box-binding proteins mentioned above) in the class II promoter region (142;153). HLA-DR, -DP, -DQ, -DM and the Invariant chain have been shown to be activated by CIITA (138;154-156). The emerging picture is that CIITA is a global regulator of the class II antigen presentation machinery and thus of the activity of CD4 T cells.

Class II, while mainly regulated by CIITA, also shows CIITA-independent inducibility under some circumstances. The CIITAKO mice have class II in the thymic epithelium (137;148;149). In addition endothelial cell class II can be induced by NK cell contact to express class II in the absence of CIITA (157). As well in some mutant cell line clones, basal DQ expression was shown to be independent of CIITA transfection (140). These few instances are the exceptions to the rule that class II expression is dependent on CIITA.

Inducible class II expression is mainly regulated at the level of transcription by CIITA (64; 66; 129; 137). CIITA has also been implicated in the translational control of class II (138; 158). CIITA itself is also regulated at the levels of both transcription and cellular localisation (159). Molecules such as IFN-γ, IL-1β, TNFα and TGFβ are implicated in the regulation of class II (160;161).

Specificity in the class II system is mediated through the multiple promoters of CIITA but also the availability of cell-specific transcription factors for class II, although those factors would be ubiquitous within each cell type (i.e. Oca-B in B cells) (162-164). Short-term changes in class II expression *in vivo* are preceded by changes in CIITA (64;165). Other transcription factors bound to the class II promoter presumably determine the *degree* of transcription of individual class II genes, making CIITA more an on-off switch for the whole class II antigen presentation system than a fine tuner of expression of each class II gene. The availability of Ii, HLA-DM (H2-M) and the class II α and β chains are regulated at least in part by CIITA (122;125;154;166-169). Together the data suggest that class II expression in non-marrow-derived tissues is regulated at multiple levels.

CIITA regulation

CIITA gene expression is regulated chiefly by IFN-γ in the induced state (138). Cells that do not express class II in the basal state, such as epithelial and endothelial cells, can upregulate class II in response to IFN-γ (14;138;170-172). This induction is mediated through CIITA (137;143;173). Recently, it was shown that CIITA is directly regulated by STAT signalling (discussed below) from IFN-γ (174). However, CIITA regulation by IFN-γ is reduced by cycloheximide treatment, suggesting that other *de novo* proteins are required for CIITA induction (64). The principal candidate is the IFN-γ response factor 1 (IRF-1). The time-course of the response to a single rIFN-γ injection shows that steady-

state mRNA levels of IRF-1 increase earlier than CIITA (15). Of significance, compared to wild-type mice, IRF-1KO mice have greatly reduced CIITA induction by systemic inflammatory stimuli and by rIFN-γ, and moderately reduced CIITA induction by injury (66). Together the data indicate that IRF-1 is required for CIITA induction.

The role of individual CIITA promoters. Multiple promoters have been described for CIITA; MHC class II expression in various cell types is correlated with differential promoter usage of CIITA *in vitro* (Figure 1.7) (175). The human and mouse CIITA 5-prime (5') regulatory regions (175-177) revealed four non-homologous promoters in the human and three in the mouse. Promoter I (PI) and promoter III (PIII) (175) are mainly detectable in dendritic-cell and B-cell lines, respectively, and are IFN-γ-independent *in vitro*. Promoter IV (PIV) has multiple potential transcription factor binding sites including γ-activated sites (GAS) and interferon stimulatory response elements (ISRE) and exhibits IFN-γ dependency *in vitro* (174;175;178). The type II promoter is detectable only in the human CIITA gene (175). Each promoter integrates messages from separate stimuli and is differentially inducible depending on cell type (175;176;179).

Recent work has identified several regulators of the CIITA promoters. Work in Ting's laboratory showed that IFN-γ inducibility is mediated through several sites in two regions close to the PIV defined by Muhlethaler-Mottet et al. (1997) (175;177;178). In human cells, signal transducers and activators of transcription 1α (STAT1α) and IRF-1 binding sites are separate and are distinct from TGF-β sites. Furthermore, PIII has also been shown to have a GAS site, at

least in human cells. TGF-β inhibits IFN-γ induced CIITA transcription (161) and IL-1β also inhibits IFN-γ induced CIITA transcription off of the PIV promoter (180). The mechanism of this suppression is unknown, but it does not appear to be competition for the IRF-1 binding site. Thus the CIITA promoters may be variably induced depending on the immune stimulus or on the tissue *in vivo*. In general, multiple promoter usage is an important mechanism of differentially mediating gene expression. In many instances the same protein is expressed in the basal state and in response to various stimuli or in spatially and temporally distinct locations, as is seen with CIITA. Thus the regulation of CIITA will be important for the immune response within a tissue since it is intrinsically related to T cell help in the tissue and draining lymph nodes.

V. IFN-γ: A KEY INFLAMMATORY CYTOKINE

It has been known for some time that MHC molecules are upregulated by IFN-γ. However, IFN-γ is involved in multiple other aspects of immunity. IFN-γ plays a pivotal role in the innate and adaptive immune responses (181). For example, IFN-γ activates macrophages to produce reactive nitrogen and oxygen intermediates, inhibits proliferation of cells and has effects on apoptosis, and displays anti-viral activity. For adaptive immunity, IFN-γ induces MHC family expression in many cell types and induces co-stimulators such as ICAM. IFN-γ upregulates chemokines such as IFN-γ-inducible T cell α chemoattractant (ITAC), monokine induced by IFN-γ (MIG), and IFNγ inducible protein 10 (IP-10)

and thereby promotes cell mediated immunity. IFN-γ also alters the cytokine balance to favour cell mediated mechanisms. IFN-γ has been shown to have anti-tumour activity (182;183) and to enhance IgG_{2a} production in B cells (184). As is obvious from this short list, IFN-γ plays multifaceted roles in immunity. In fact, over 200 genes are known to be regulated by IFN-γ (184).

A. IFN-γ and Its Receptor

The homodimeric cytokine is encoded by a single gene and is 143 a.a. in length (184). The IFN-γ receptor is a heterodimer consisting of the high affinity α-chain (90 kDa; $Ka=10^9$-10^{10} /M) which interacts with the ligand (181;184). The low affinity β-chain is 314 a.a. and is required for signalling. The receptor is ubiquitously expressed on nucleated cells with its highest numbers on non-lymphoid cells. IFN-γ is secreted by CD4 and CD8 T cells and NK cells and its secretion is induced by mitogens and LPS stimulation as well as by antigen recognition by T cells. To generate its multiple effects, a homodimer of IFN-γ assembles the α-chains of the IFN-γ R complex (Figure 1.8). β-chains with JAK2 are then recruited to the complex. JAK1, associated with the α-chain, is phosphorylated on tyr440 after JAK2 is assembled. Phosphorylation and thus activation of the JAKs recruits to the complex the SH2-domain containing signal transducers and activators of transcription (STAT) molecules which are then phosphorylated on tyr701 (185-188). STAT 1α then dimerises, translocates to

the nucleus, and becomes an active transcription factor that binds to gamma activated sites (GAS) in target promoters. The levels of STAT 1α are regulated by ubiquitination and IFN-γ and its receptor complex are internalised after signalling (184).

The IRF-1 Transcription Factor Family. IFN-γ and its receptor regulates various transcription factors. The primary response is STAT activation and secondary responses include activation of the interferon regulatory factor (IRF) family of transcription factors. The IRF family is growing, but includes the well-studied members IRF-1, IRF-2, ICSBP, IRF-3, IRF-7, Pip, LSIRF and p48 (ISGF3γ) (184;189-192). The family members share homologies in their DNA binding domains and bind the same interferon-stimulated response element (ISRE). A GAS site mediates IRF-1 expression and IRF-1 is synergistically inducible by IFN-γ and TNFα.

B. IFN-γ Regulates Antigen Presentation.

IFN-γ regulates the expression of MHC products and other components of the antigen presenting machinery such as TAP, LMP, PA28, invariant chain, $\beta_2$microglobulin, and cathepsin S (112;193-196). Regulation of the antigen presentation machinery by IFN-γ involves first wave transcription factors (STAT1) and second wave transcription factors like IRF-1. All known downstream effects of IFN-γ on antigen presentation are mediated by these factors.

Like other cytokines and growth factors, IFN-γ may be expressed in low levels in normal hosts, and thus could play a role in basal gene expression, as well as during acute responses and disease states. IFN-γ has both unique and redundant functions and the availability of mice with disrupted IFN-γ genes (GKO) or with disrupted IFN-γ receptor (GRKO) genes permits redundancy and uniqueness to be examined definitively. GKO have defects in responses to infectious agents and have excessive proliferation of splenocytes in culture in response to stimuli (197). Mice with disrupted IFN-γ receptor genes have defective nitric oxide production, decreased IgG_{2a} production, and decreased susceptibility to LPS (198-200). STAT1α KO mice carry a similar phenotype as the IFN-γ receptor KO mice (201) indicating that STAT1 plays an important role in mediating IFN-dependent responses.

MHC class I is controlled by IFN-γ through its 5' ISRE by IRF-1 (191;202-204) although studies first done in the lymphocytes from the IRF-1 KO indicated that class I was not dependent on IRF-1. However in non-lymphoid tissues, class I induction is dependent on IRF-1 *in vivo* (15;205) (Figure 1.4). IFN-γ is thought to synergise with TNFα for class I induction and the ISRE and NF-κB sites are required for the effects, at least in human cell lines (206-208). Furthermore, it was shown that co-transfected IRF-1 and p65 of NF-κB interacted and activated constructs containing enhancer A and the ISRE of class I (209). β2-microglobulin is also regulated by IFN-γ although its promoter is unrelated in structure to class I (208). The promoter contains an ISRE and a NF-κB site and

it is thought that these elements allow it to be regulated in a parallel manner to class I (184).

Both chains of TAP map to the *MHC class II* region and are inducible by IFN-γ. Constitutive and inducible levels of TAP correlate with class I expression. IFN-γ also regulates LMP2, 7 and 10; the levels of these proteasomal subunits are low in the resting state (210;211). Both LMP2 and 7 map to the *MHC class II* region (LMP10 maps outside the *MHC* region) and are relatively close to the TAP genes. Of interest, the regulation of *LMP2* and *TAP1* genes is co-ordinated and the two genes share a common bi-directional promoter (98). Furthermore, these genes are potentially regulated by IRF-1 and the genes contain a GC box (transcription factor SP-1 binding site), a GAS site and an imperfect NF-κB site (210).

The proteasome activator (PA28) heterodimer and Tapasin (gp48, discussed in section IV.A) are also regulated by IFN-γ. PA28 assembles as a ring on the end of the core proteasome and enhances the efficiency of double endopeptidase cleavages to increase the yield of peptides that TAP can bind and load onto class I (81;82). Tapasin and ERp99 (gp96) are also regulated by IFN-γ. ERp99 is one of several chaperones that are implicated in the protection and carrying of peptides to be loaded onto class I (212).

Many of the members involved in class II antigen presentation (α- and β-chains, Ii, H-2M) are encoded in the *MHC* region and are inducible by IFN-γ. The promoters of these class II-related IFN-γ inducible genes share a similar architecture (213;214) and several common factors regulate the expression of

the genes. These factors include IRF-1, CIITA, RFX, and NFY. Of these factors, IRF-1 and CIITA are regulated directly by IFN-γ through STAT1.

One area in which IFN-γ has a unique role is the regulation of MHC expression, both in the basal and induced states. We have previously reported that a component of basal MHC expression in mouse tissues may be cytokine-induced, possibly by IFN-γ (215). We also showed that ischemic injury induces MHC expression locally (63;65) and that a variety of potent inflammatory stimuli, such as allogeneic cells, skin sensitisation with oxazolone, or LPS, induce increased systemic MHC expression in nonlymphoid organs such as kidney and heart (216;217). From data taken from tissue sections, this increase occurred mainly in parenchymal and endothelial cells. These stimuli trigger IFN-γ production, and the induction of MHC class I and II expression is inhibited by anti IFN-γ mAb and by cyclosporine. However, it has been impossible to exclude the participation of other cytokines. Similarly, complex processes such as graft rejection invariably have increased MHC expression, probably reflecting the combined action of locally acting and systemic stimuli (218;219). My experiments presented in this thesis will address the role of IFN-γ in MHC levels in the basal and induced states.

VI. RATIONALE AND HYPOTHESIS

Clinical and rodent data show that MHC molecules are involved in kidney rejection. MHC-mismatched kidneys are rejected more efficiently by the host immune system than matched kidneys. Furthermore, MHC molecules are upregulated in the kidney in response to systemic inflammation and local injury. Mechanisms that induce MHC expression in kidney are likely to be involved in the mechanisms of transplant rejection and acceptance, and therefore tolerance induction. I studied renal MHC regulation in the kidney in a mouse model. I examined MHC regulation in the basal state, in response to various systemic stimuli and in response to injury and transplantation. Since antigen recognition is intimately involved in the immune response *in vivo* I hypothesised that MHC induction in the kidney would be involved in the response to systemic inflammation, injury and transplantation. To address this hypothesis I proposed several questions.

1. What is the role for IFN-γ in MHC regulation in the kidney?
2. Is the antigen presenting machinery upregulated in the kidney?
3. How does CIITA in mouse differ from the human?
4. Is CIITA involved in MHC regulation in kidney?
5. How is CIITA regulated *in vivo* in the kidney?

I attempt to address these questions in the following chapters. The answers contribute to the body of knowledge for MHC regulation in the kidney and also may be applicable to other non-lymphoid organs in various disease states *in vivo*.

VII. Table

Table 1.1: Differences between class I and II systems of antigen presentation
The differences are thought to be responsible for the different peptide pools bound by MHC molecules.

	Class I	Class II
Components:	heavy chain + beta2-microglobulin	alpha + beta chains
Co-receptor binding:	CD8	CD4
Responding cell:	CD8+ CTL	CD4+ T helper
Peptide binding groove:	closed	open
Peptide size:	8-11 amino acid	12-22 amino acid
Locale of peptide loading:	in ER	in endosomal pathway
Source of peptides:	cytosol/nucleus	endosome, membranes, serum
Anchoring to groove:	allelic specific motifs	core sequence with variable ends
Cells expressing MHC:	all nucleated cells	APC and IFN-γ-induced cells

VIII. FIGURES

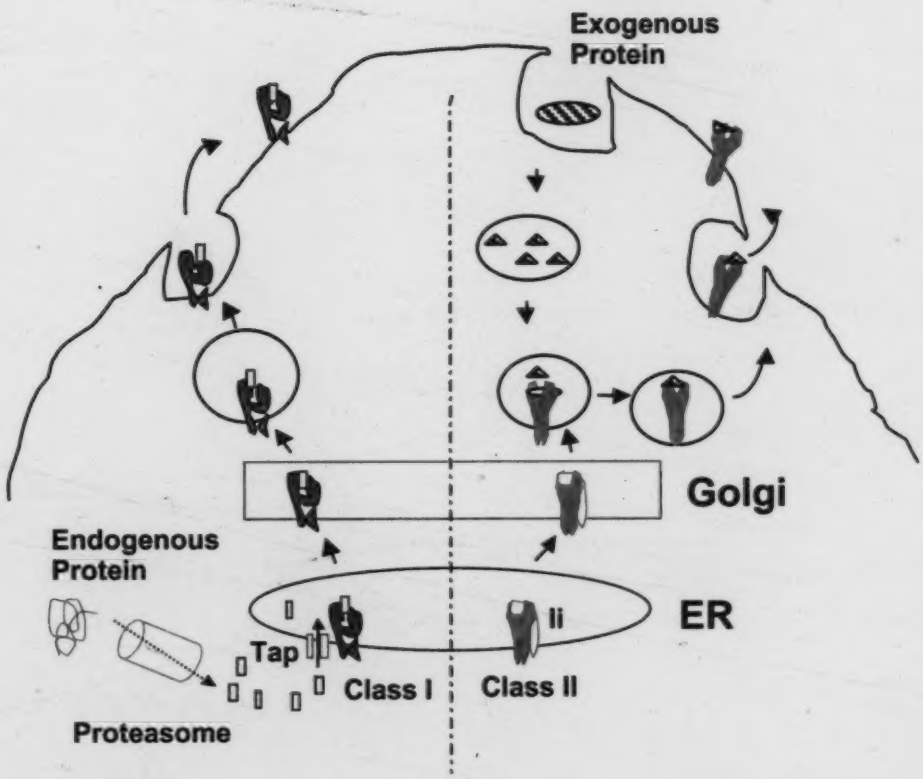

Figure 1.1: APCs present both endogenous and exogenous peptide pools on MHC molecules. For class I, peptides are generated by the proteasome and enter the endoplasmic reticulum (ER) via the Tap transporter. Class II encounters peptides in the endosome.

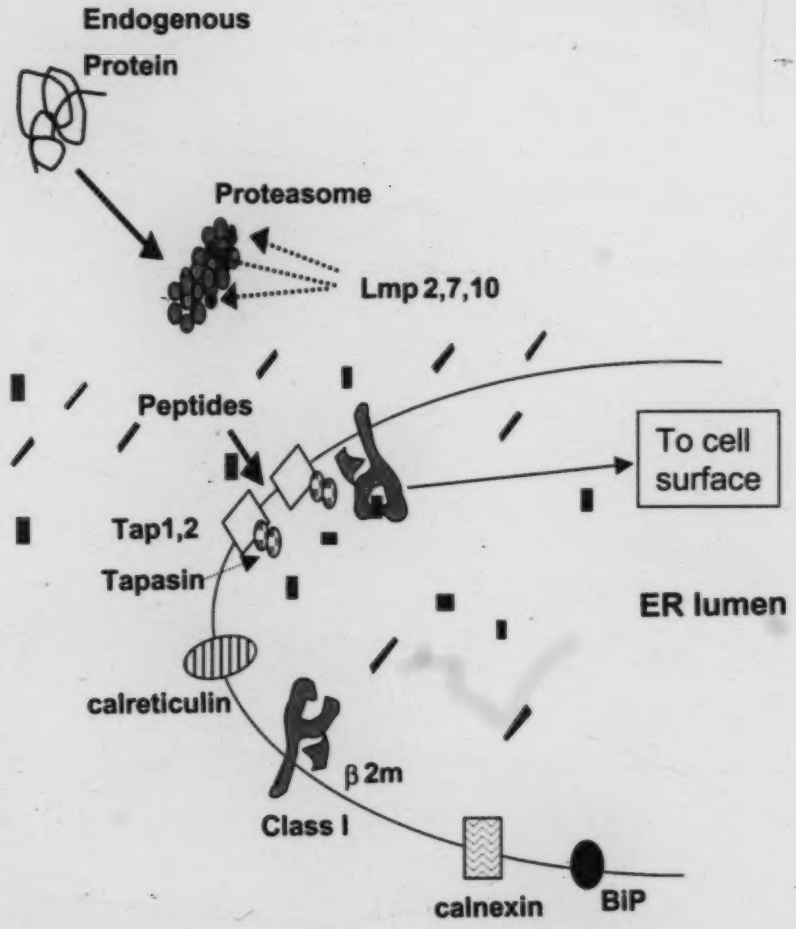

Figure 1.2: Class I requires interactions with multiple chaperones to introduce peptides to class I molecules. Once the heavy chain is loaded with peptide, it can traffic to the cell surface. Details of the interactions are in the text.

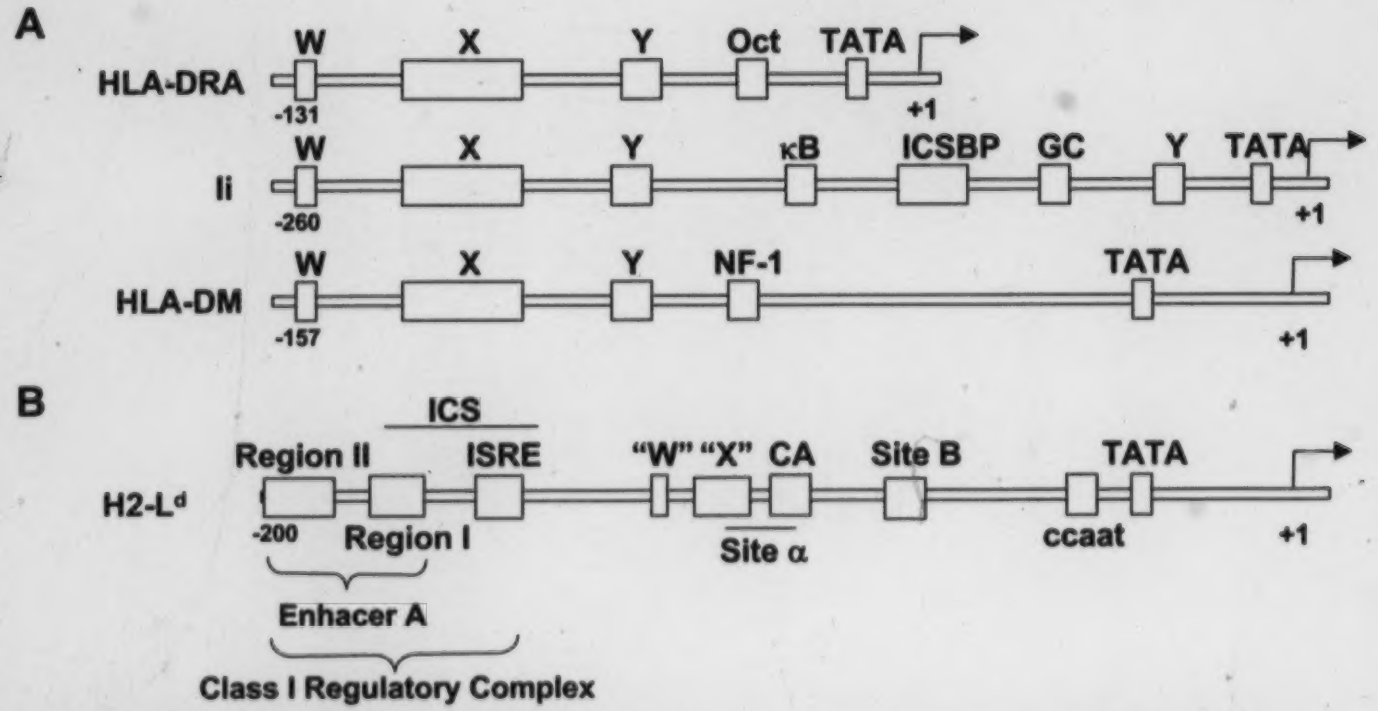

Figure 1.3: Conserved architecture of the MHC family 5' regulatory regions. **(A)** Class II family members have conserved architecture in their 5' region including the W,X and Y boxes. Other proposed binding sites in the family members are also shown. ICSBP, IFN consensus sequence binding protein **(B)** Class I genes share common regulatory elements. ISRE is also called the IRE (IFN response element). W and X sites are analogous to the class II regions ICS, IFN consensus site.

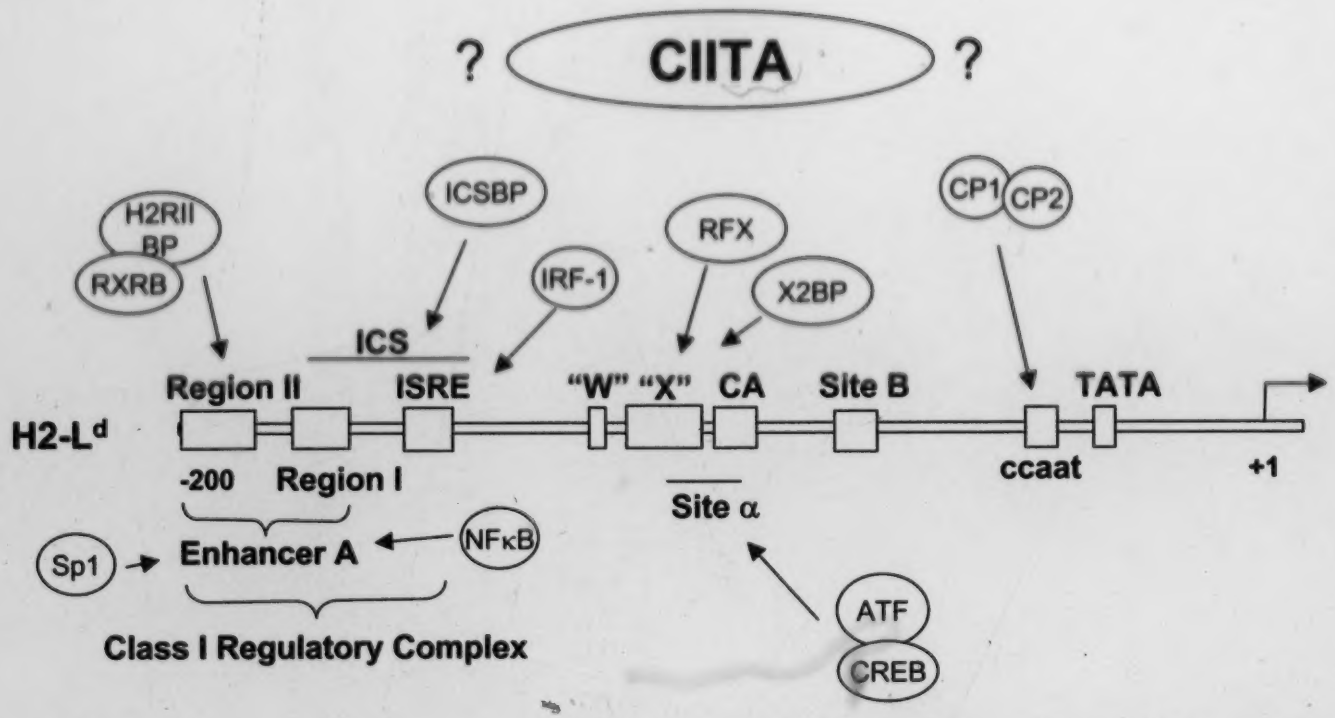

Figure 1.4: Proven and proposed regulatory sequences of the class I 5' regulatory region. ICS, IFN consensus sequence; ISRE, IFN stimulated response element; H2RIIBP, H2 region II binding protein; IRF-1, IFN regulatory factor-1. W and X boxes are proposed sites adapted from class II promoter regions based on homologous sequences. It is proposed that CIITA activates class I promoters via these proposed W and X sites .

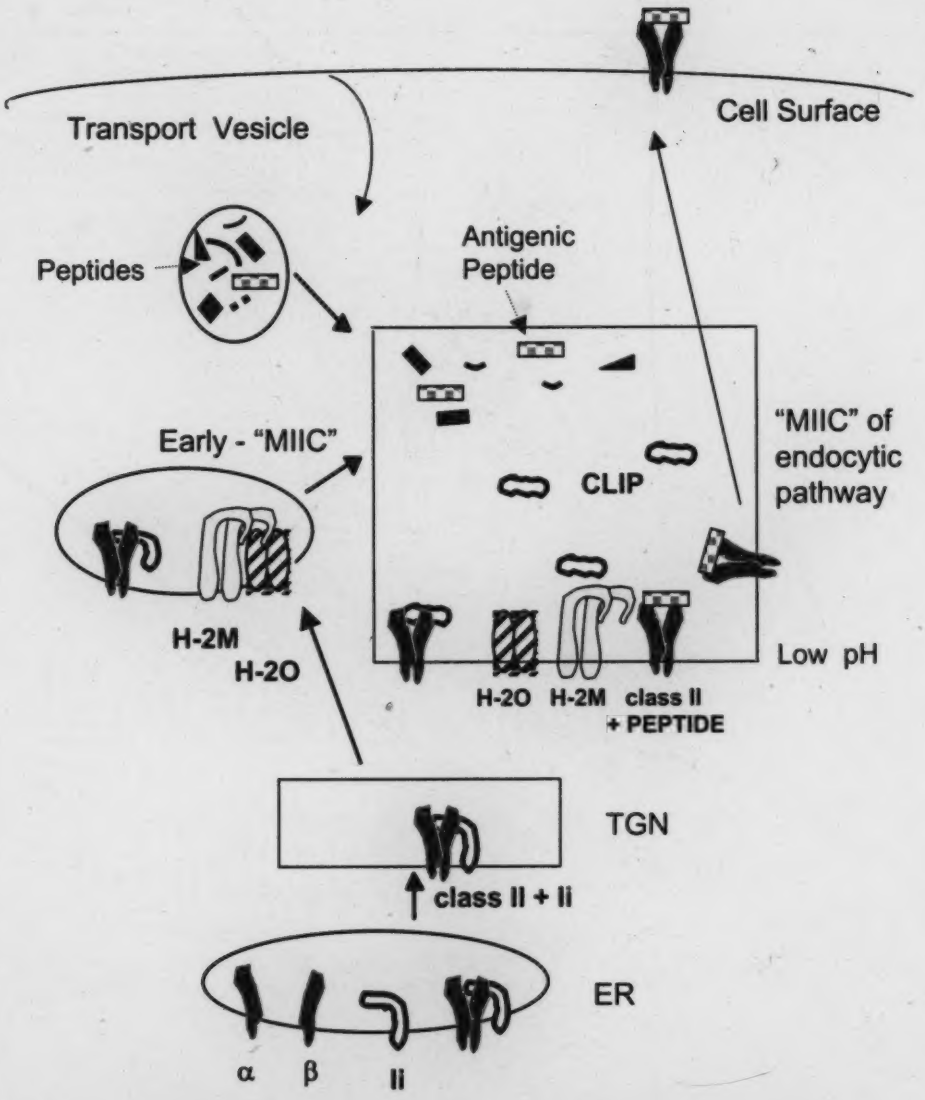

Figure 1.5: The endocytic pathway to introduce peptides onto class II molecules. The Ii:class II nonamer is simplified to a trimer. TGN, trans-golgi network; ER, endoplasmic reticulum; MIIC, MHC class II compartment. Not to scale.

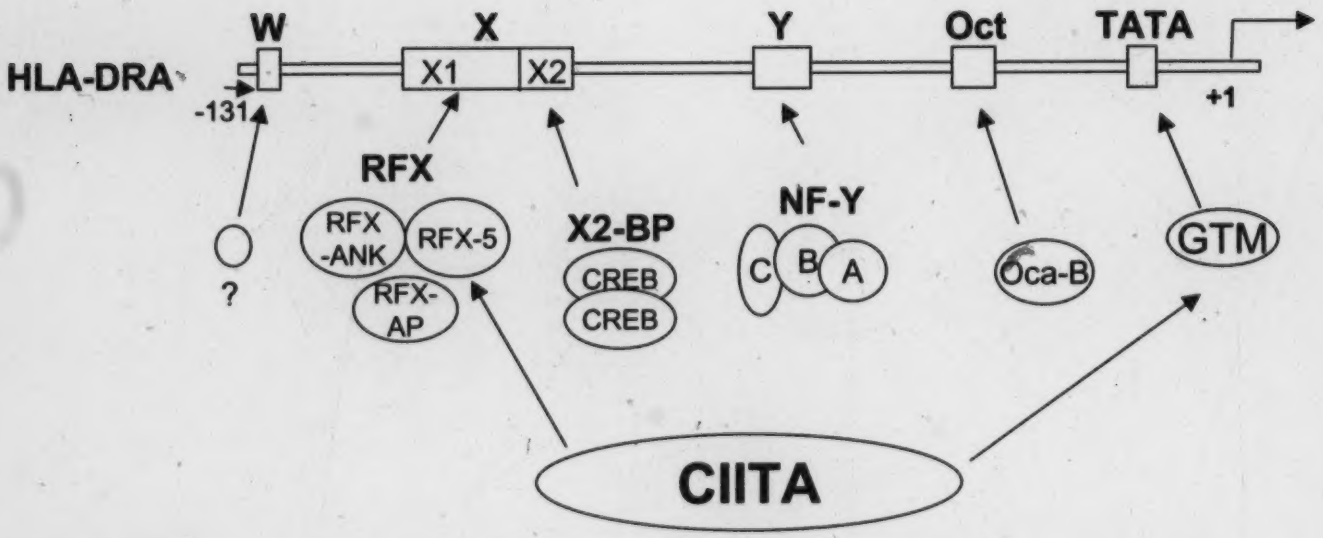

Figure 1.6: Class II promoter structure with conserved sites (W, X box, Y box, Oct, and TATA box) for transcription factor binding. CIITA has been shown to interact with members of the GTM and RFX-5 in human. It is unknown which transcription factors bind to the W box. GTM, general transcription machinery

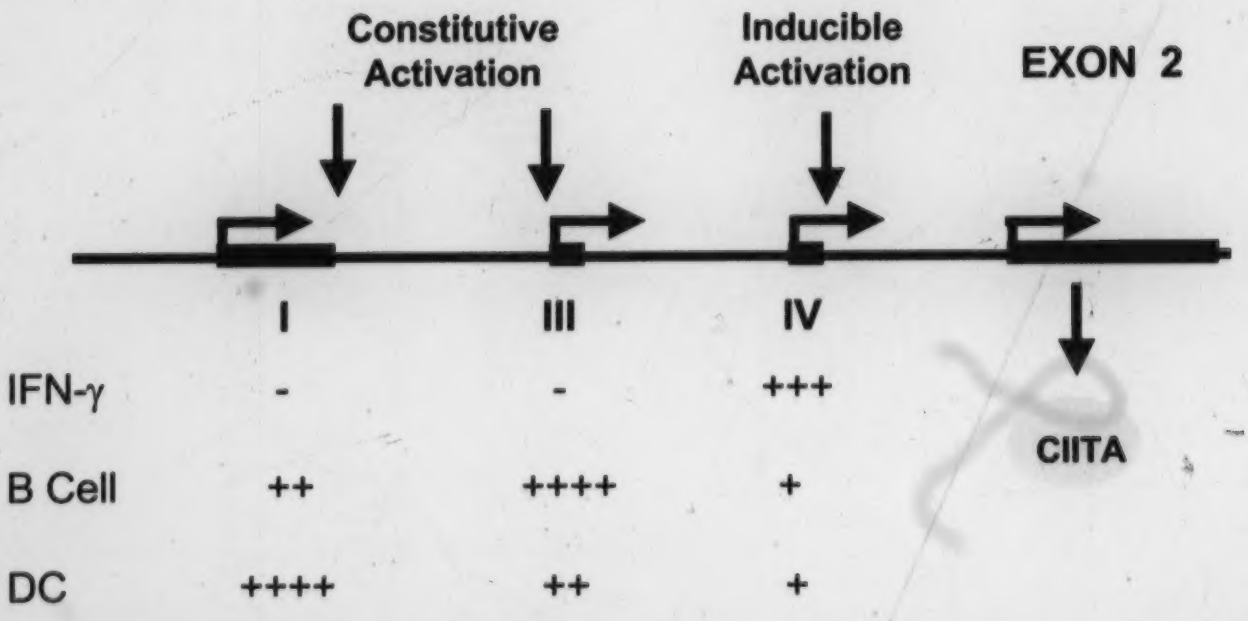

Figure 1.7: Genomic arrangement of the mouse CIITA promoters and Exons 1. Promoters I and III are proposed to be responsible for constitutive CIITA and class II expression, while Promoter IV is thought to be involved in IFN-γ–inducible activation.The predicted expression patterns of each promoter is also noted. Dendritic cell (DC).

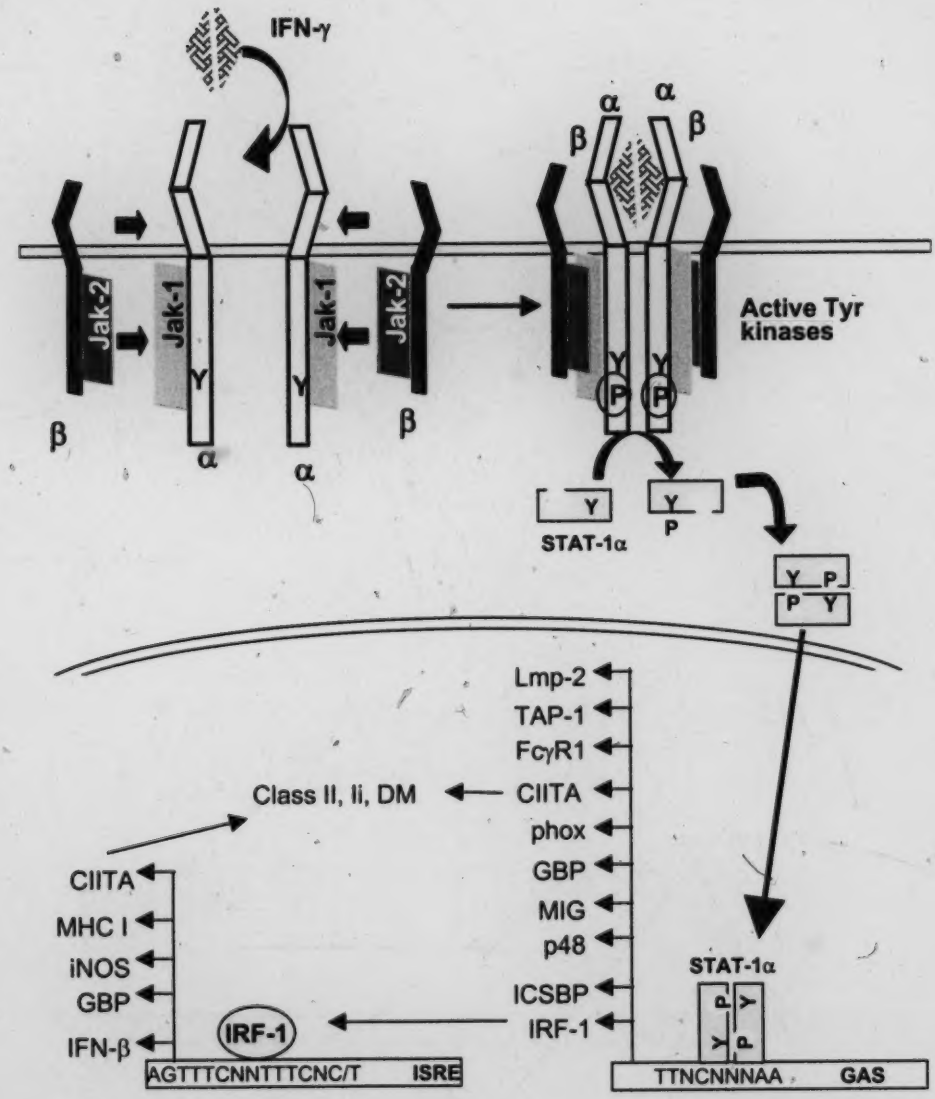

Figure 1.8: IFN-γ dimerises its receptors (α, β chains) activating the kinase (JAK)-STAT pathway. STAT1α transcription factor enters the nucleus and activates target genes including other transcription factors like IRF-1.

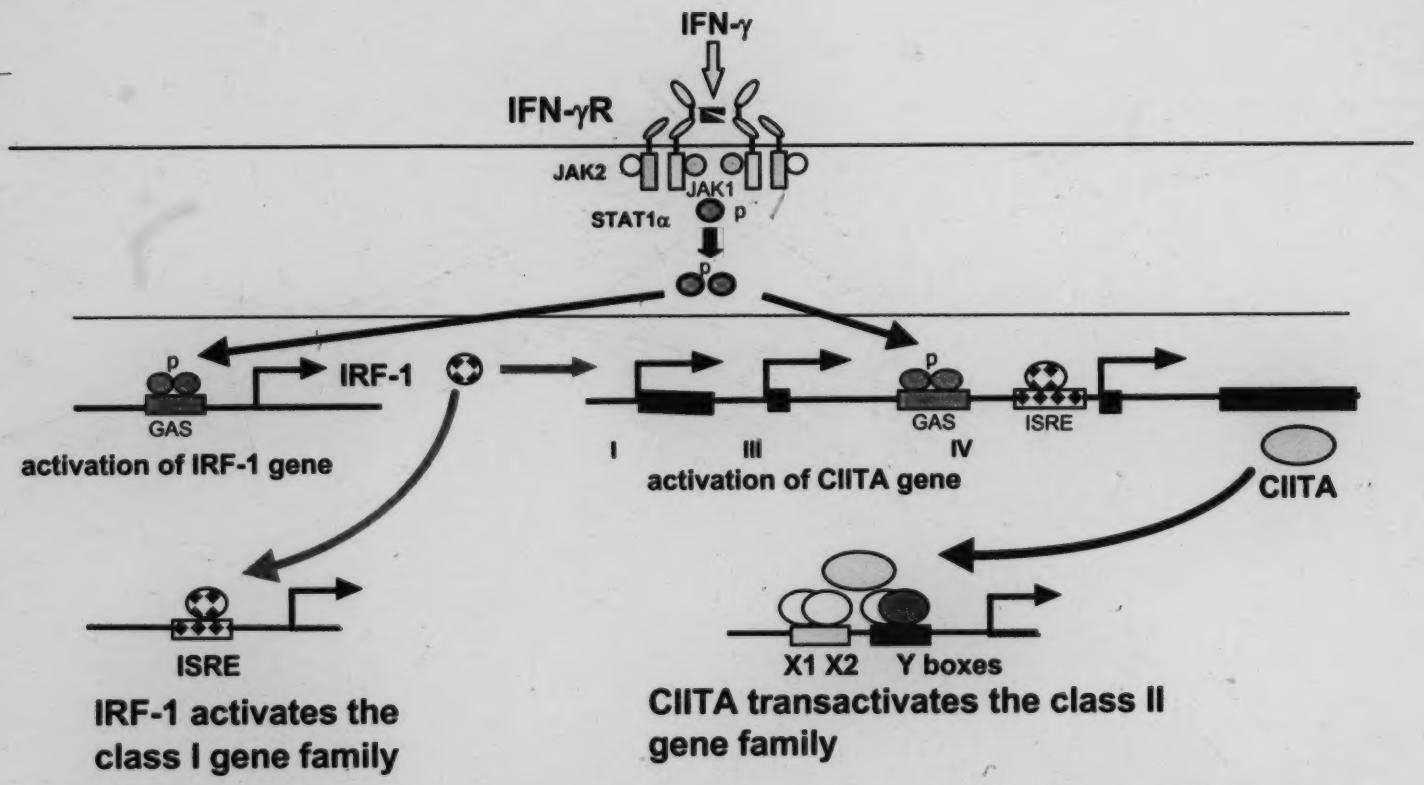

Figure 1.9: IFN-γ induces antigen presentation through conserved gamma activated sites (GAS) in target genes. The targets themselves may be transcriptional activators such as IFN regulatory factor (IRF-1) that binds the ISRE and CIITA. Other transcription factors not shown bind each promoter .

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CHAPTER 2

Basal and Induced MHC Class I and Class II Expression:

Dependence on IFN-γ

Chapter 2

Basal and Induced MHC Class I and Class II Expression: Dependence on IFN-γ

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I. PREFACE

It had been thought that IFN-γ played an important role in MHC induction, but no studies had delineated the role of IFN-γ *in vivo* using genetic evidence. I therefore set out to study MHC regulation in the kidney in the IFN-γ gene-disrupted mice under various conditions that were known to increase MHC levels in the kidney.

II. INTRODUCTION

In the present studies I compared MHC expression in IFN-γ gene-disrupted (GKO) and wild-type (WT) mice. I assessed the role of IFN-γ in two states of MHC expression: basal and systemically induced. In the basal state, GKO mice had reduced class I expression in many tissues, particularly in arterial endothelium, but no reduction in class II. The systemic MHC induction was mediated uniquely by IFN-γ.

III. MATERIALS AND METHODS

GKO mice. The original GKO mice were created by disrupting the IFN-γ gene, inserting a neomycin-resistance gene (*neo*^{r}), and replacing one copy of the wild-type gene in embryonic stem cells by homologous recombination. These stem cells were used to construct mice heterozygous for the disrupted gene, which were intercrossed and the progeny were selected for homozygosity (1). Heterozygous BALB/c and BALB/c mice with intact wild-type IFN-γ genes were provided to us as a generous gift from Dr Tim Stewart (Genentech Inc., South San Francisco, CA). The backcrossed mice were genotyped by PCR amplification of tail skin DNA using two set of primers for the gamma gene and for the neo insert (2). The first set amplifies a band of 220 bp on the intact IFN-γ gene and the second set amplifies a band of 375 bp on the region of the targeted mutation (*neo*^{r}). The PCR was run for 40 cycles in a Perkin Elmer Cetus thermocycler (Norwalk, CT) using Taq DNA polymerase (BRL, Burlington, Ontario, Canada) at

55°C annealing temperature. The PCR product was run on an ethidium bromide gel, the GKO homozygotes showed a single (*neo*^{r}) band, the wild-type a single IFN-γ band and the heterozygous showed the two bands. The heterozygotes were intercrossed (Health Sciences Laboratory Animal Services) and the homozygous GKO mice were identified by tail skin DNA testing. Homozygosity of the offspring was confirmed by further tailskin testing, and by testing each mouse for IFN-γ mRNA expression by RT-PCR on spleen RNA at the time of experimental use. All GKO mice used were confirmed homozygotes (confirmation done by Joan Urmson).

Wild-type mice. BALB/c mice with wild-type IFN-γ genes aged 6-12 weeks were obtained from Jackson Laboratories, Bar Harbor, ME, (BALB/cByJ), from Charles River, Montreal, Canada (BALB/cAnNCrlbr), or from the Health Sciences Laboratory Animal Services at the University of Alberta (BALB/cCr//AltBM). The mice were maintained in the HSLAS animal colony and were kept on acidified water. All experiments conformed to approved animal care protocols.

IFN-γ inducing stimuli:

LPS injections. *Salmonella minnesota* LPS was dissolved in sterile saline at 250 μg/ml and heated at 60°C for 5 hours. Mice received 25 μg i.p. on day 0 and were killed at 72 hours.

Recombinant IFN-γ (rIFN-γ) injections. Mice received 100,000 I.U. of rIFN-γ, a generous gift from Dr. Peter van der Meide (Institute of Applied

Radiobiology and Immunology, Rijswijk, The Netherlands) i.p. on day 0 and were sacrificed 72 hours later.

Antibodies. Hybridoma cell lines were obtained from ATCC (Rockville, MD) and the cell lines producing mAb 25-9-17SII (anti I-A^d), 34-4-20S (anti H-2D^d), M1/42.3.9.8 (anti H-2 antigens all haplotypes) and M5/114.15.2 (anti I-$A^{b,d,q}$ and I-$E^{d,k}$) were maintained in tissue culture in our laboratory. The supernatants containing 25-9-17SII (anti I-A^d) and 34-4-20S (anti H-2D^d) were purified by protein A chromatography. M1 and M5 were ammonium sulphate precipitated and then further purified using a DE52 anion exchanger column (Whatman, Hillsboro, OR), and concentrated by Amicon ultrafiltration. The protein concentration was determined by a modified Lowry method, adjusted to 1 mg/ml and the stock solutions were kept frozen at -70°C. Radioiodination was performed by Iodogen method (Pierce Chemical Co., Rockford, IL) (3). Anti IFN-γ (R4-6A2) (4) mAb was injected i.p. (100 μg) each day for 7 days, starting one day before the injection of P815 cells.

Radiolabelled antibody binding assay (RABA). Radiolabelled antibody binding assays are done exclusively by Joan Urmson. This technique has been previously reported (5;6). Briefly, tissues of individual mice were homogenised by polytron in 1 ml PBS in preweighed Sorvall tubes, washed in 10 ml PBS and centrifuged at 3000 rpm (2000 x G) for 20 minutes. The supernatants were discarded and the tubes were re-weighed. The pellets were polytroned again in 1

ml PBS and the tissue concentration was adjusted to 20 mg/ml. Five mg of kidney, 2.5 mg of liver and 10 mg of heart tissue were aliquoted in triplicate and spun at 3000 rpm for 20 minutes. The pellets were resuspended in 100 μl of radiolabelled mAb in 10% normal mouse serum (100,000 cpm per 100 μl) and were incubated on ice with agitation for 60 minutes. One ml of PBS was added to all of the tubes and spun at 3000 rpm for 20 minutes. The pellets were counted in a gamma counter and the non-specific binding of a negative tissue was subtracted. Statistical significance was determined using ANOVA, $p < 0.01$ (SPSS programme).

Staining of tissue sections. Fresh frozen cryostat sections were fixed in acetone, then incubated with normal goat serum. The slides were then incubated with mAb anti class I (M1) and class II (M5) or controls followed by affinity purified peroxidase labelled goat anti mouse IgG or goat anti rat IgG $F(ab')_2$ fragment (Organon Teknika Inc., Scarborough, ON). The slides were incubated with 3'3 diaminobenzidine tetrahydrochloride and hydrogen peroxide for the colour reaction and then counterstained with hematoxylin. Joan Urmson provided technical assistance with this tissue staining.

Northern blot analysis. Total RNA was extracted from pooled samples according to a modification of the method described by Chirgwin (7). Tissues were homogenised one at a time with a polytron in 4 M guanidinium isothiocyanate on ice and spun at 10,000 rpm (Sorvall, RC-5B) for 10 min. The supernatants were

layered over a 5.7 M $CsCl_2$ cushion. RNA was pelleted through the cushion on a Beckman ultracentrifuge at 50,0000 rpm for 4-5 hours. RNA concentrations and purity were determined by absorbance at 260/280 nm. Northern blots were prepared by pooling equivalent amounts of RNA from each sample and using 5-10 μg of total RNA electrophoresed through a 1.5% agarose gel in the presence of 2.2 M formaldehyde. The gels were transferred to nitrocellulose filters and put in prehybe solution (8). cDNA probes for the HLA-A3 gene (class I) and with β-actin for loading control were labelled using 1μl Klenow (Roche, Laval, QC) and 10μl [^{32}P]αdCTP. Blots were than hybridised overnight at 42^{0}C. Blots were washed to 0.2X SSC+SDS. Kodak X-Omat AR film was exposed to each blot, with an intensifying screen, at -70°C.

Reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was prepared by the guanidinium-cesium chloride method as described above and then reverse transcribed into cDNA using Superscript reverse transcriptase (BRL, Burlington, ON). The cDNA was amplified in a Perkin Elmer Cetus thermal cycler using Taq polymerase and IFN-γ specific oligomer primers. The IFN-γ sequence and PCR method have been previously described (9). The reverse transcription was incubated at 42°C for 45 minutes and then 10 min at 97°C. The PCR was run for 30 cycles at 52°C annealing temperature. The IFN-γ primers amplified a 434 bp fragment on an ethidium bromide gel, recognised by a specific mouse IFN-γ cDNA probe (generously provided by Genentech, South San Francisco, CA) on Southern blot.

IV. RESULTS

Basal MHC expression in kidneys of GKO mice. We assessed MHC product expression by RABA in kidney homogenates of 36 wild-type and 36 GKO mice, age and sex matched (Table 2.1). Basal class II expression was equal in GKO and in the wild-type mice. However, class I expression was 40% less in GKO than in wild-type mice. In tissue sections from unstimulated wild-type mice, the arterial endothelium was the only site that stained strongly for class I (Figure 2.1 top panel, arrow); the parenchymal cells and interstitial cells were negative or weakly positive. In the GKO mice (Figure 2.1 bottom panel), the class I staining of arterial endothelium was reduced or absent, and no parenchymal staining was seen. Looking at Figure 2.2, the MHC class II molecule staining was equal in wild-type (top) and GKO (bottom) kidneys and was confined to interstitial cells (arrow). The endothelium and glomeruli were also negative for class II in both wild-type and GKO mice. Northern blots also showed that class I mRNA was reduced in GKO mice (see sham injected mice in Figure 2.4, below), but class II was similar to the wild-type mice.

The effect of various IFN-γ-inducing stimuli on systemic MHC expression.

The response of GKO mice to LPS. LPS can induce a wide range of biological activities in macrophages, such as 1) induction of TNFα, IL-1α and IL-1β; 2) internalisation of TNF-α receptors; 3) induction of some immediate early

genes; 4) induction of nitric oxide production; 5) and induction of MAPK family and lyn kinase of the T cell receptor pathway. LPS also induces MHC expression in many tissues, but unlike oxazolone and allogeneic stimulation, LPS is equally effective in normal and T deficient mice (10;11). By radiolabelled antibody binding assay, wild-type mice given LPS showed a large increase in MHC expression by day 3 (Figure. 2.3), whereas GKO mice showed no increase in class I or class II expression. By northern blot (Figure 2.4) RNA levels are also increased in response to LPS in wild-type but not in GKO mice.

Looking at the immunohistochemistry of all MHC molecules distributed within the tissue, class I molecules were induced on the tubular epithelium of the wild-type mice (Figure 2.5, top panel) but no class I was stained in the GKO mice (Figure 2.5, bottom panel). MHC class II molecules (Figure 2.6) were induced on the tubular epithelium of wild-type mice (top, large arrow) but no induced staining was seen in the GKO mice (bottom). However, the interstitial cells remained positive as in the basal state in the GKO mice (small arrow). Thus the systemic MHC response to LPS injection is uniquely due to IFN-γ.

The response to rIFN-γ. I also compared the responses of GKO and wild-type mice to an intraperitoneal injection of 100,000 U of rIFN-γ at day 3 (Figures 2.3 and 2.4). This control had two purposes: first, to establish whether IFN-γ receptor and the system for inducing MHC expression was intact in GKO mice; and second, to test whether autoinduction of endogenous IFN-γ amplifies the response to exogenous rIFN-γ as previously suggested (10;12). I found that

exogenous rIFN-γ induced kidney MHC class I and II expression equally in GKO and wild-type mice. Tissue staining for class I (Figure 2.7, top and bottom) and class II (Figure 2.8, top and bottom) showed strong induction on the tubules in both wild-type and GKO mice, respectively. The arteries in both types of mice remain negative for class II even with rIFN-γ stimulation. Thus both types of mice are able to respond to rIFN-γ and the mice do not require endogenous IFN-γ to upregulate MHC at remote sites.

V. DISCUSSION

The availability of GKO mice permitted us to dissect the role of IFN-γ in normal and induced MHC expression under circumstances where the rest of the cytokine responses are intact. GKO mice had normal basal class II expression, attributable to interstitial cell staining in their kidneys but the level of class I mRNA and product was reduced as was the class I staining of arterial endothelium. Thus a component of basal class I expression requires IFN-γ, but class II expression in interstitial cells does not. Stimuli that induce potent IFN-γ responses (i.e. LPS, shown here, or P815 or oxazolone (2)) strongly induce systemic MHC class I and II expression in many tissues of wild-type mice, but had no effect on systemic MHC expression in GKO mice. Thus systemic MHC induction in response to inflammatory stimuli is a unique and non-redundant role for IFN-γ.

Basal MHC class I expression, unlike class II, is partially IFN-γ dependent. Class I is expressed abundantly on arterial endothelium and diffusely at low levels in many parenchymal cells. The present results indicate that some of the class I expressed *in vivo* is in fact not constitutive, as previously assumed, but is dependent on IFN-γ. This seems particularly true for arterial endothelium. These data suggest that an IFN-γ-induced factor such as IRF-1 (13) is needed to activate the class I promoter even in the basal state.

My finding that GKO mice have normal basal class II expression in interstitial cells in kidney is consistent with a requirement for local stimuli (i.e. cytokines or cellular matrix) to induce class II expression, but rules out any obligatory role for IFN-γ. In most mouse tissues, basal class II is confined to interstitial cells of dendritic morphology. These are not strictly defined dendritic cells: renal class II positive cells are neither homogeneous nor functionally competent dendritic cells, although some become competent dentritic cells in culture (14). The class II positive interstitial cells in various organs are probably not constitutively class II positive, but are induced by local non-IFN-γ stimuli in their environment. In culture, hepatic "dendritic cells" differentiate from class II negative precursors to be class II positive in the presence of GM-CSF plus some components of the connective tissue of liver, but do not require IFN-γ (15). Experiments with class II transgenes with mutated promoters show that class II positive interstitial cells are not constitutively positive like B cells (16). Thus it is likely that locally operating tissue specific signals induce class II expression in interstitial cells.

IFN-γ, whether administered exogenously or induced endogenously, is uniquely able to induce a systemic increase in MHC expression. We have administered IFN-α, TNF-α, GM-CSF, IL-10, IGF-1, and EGF to mice and failed to see a significant increase in systemic class II expression, and with the exception of IFN-α have seen no increase in class I (11;17;18). The failure of GKO mice to show systemic MHC induction in response to LPS and other stimuli, all of which are potent inducers of many cytokines, including TNF-α, confirms the unique role of IFN-γ in systemic MHC induction. The fact that IFN-γ receptor deficient mice are resistant to endotoxic shock indicates that IFN-γ may have other unique systemic roles (19). Although IFN-γ induces local IFN-γ mRNA production in some sites (20;21), the present experiments show that the response to exogenous IFN-γ is not impaired when the host cannot make IFN-γ, arguing that autoinduction is not important in the systemic effects of IFN-γ. Perhaps the mRNA induced by IFN-γ leads to relatively little useful product. Amplification by autoinduction may still play a role in other circumstances.

IFN-γ plays unique and non-redundant roles in MHC regulation, including basal class I expression and systemic MHC induction in response to strong stimuli. The uniqueness of IFN-γ in systemic MHC induction may be attributable to the IFN-γ receptor and signalling system being in a state of perpetual readiness in many normal tissues, whereas other cytokines may lack receptor in most parenchymal cells, or require induction of receptor or signalling components before response can occur. Such induction may only occur in the context of local injury or

inflammation, which could account for the ability of non IFN-γ stimuli to induce local MHC expression in tissue injury but not a systemic response (2). Alternatively stimuli that induce class I and II expression in GKO mice may only be released locally, and may not be released systemically. While further experiments are needed to answer these questions, the present results demonstrate that basal and systemic MHC expression represent distinct programs.

VI. TABLE

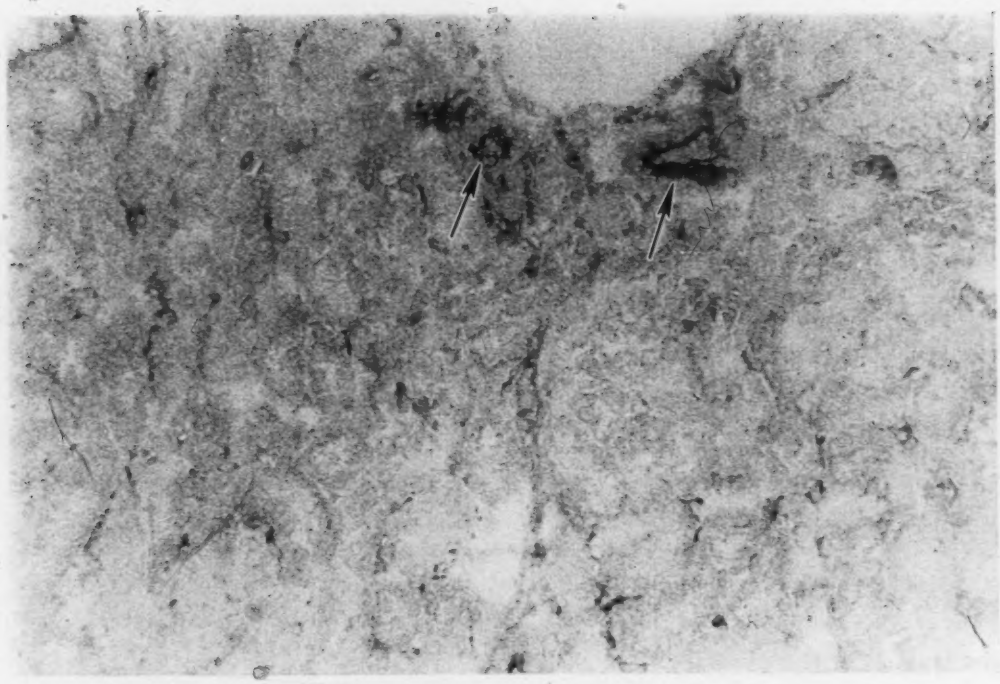
Table 2.1: MHC class I ($H\text{-}2^d$) and II ($I\text{-}A^d$ and $I\text{-}E^d$) detection in sham treated kidney homogenate using radiolabelled monoclonal Ab. The results are expressed as mean ± SD in each experiment, and mean ± SD of the pool of all mice in the experiments*.

	Mice	Class I	Class II
Expt 1*			
(3 mice, 8 weeks)**	Wild^	4836 ± 1221	1391 ± 595
	GKO	1621 ± 64	1025 ± 163
Expt 2*			
(3 mice, 13 weeks)	BALB/cAnNCrlbr	4087 ± 1432	674 ± 837
	GKO	2532 ± 346	566 ± 207
Expt 3*			
(6 mice, 12 weeks)	Wild	2393 ± 399	2392 ± 399
	GKO	2307 ± 339	2306 ± 339
Expt 4			
(5 mice, 12 weeks)	Wild	1005 ± 140	568 ± 161
	GKO	680 ± 221	639 ± 236
Expt 5*			
(5 mice, 10-12 weeks)	BALB/cAnCrlbr	2347 ± 485	619 ± 496
	GKO	1901 ± 306	601 ± 432
Expt 6*			
(5 mice, 8 weeks)	BALB/cAnCrlbr	1564 ± 189	147 ± 349
	GKO	1494 ± 530	399 ± 632
Expt 7			
(6 mice, 8 weeks)	Wild	1594 ± 1012	434 ± 320
	GKO	1001 ± 291	542 ± 350
Expt 8			
(3 mice, 8-10 weeks)	BALB/ccCrl/AltBM	1814 ± 98	950 ± 481
	GKO	613 ± 184	222 ± 360
TOTAL: Wild-type	**(n= 36)**	2242 ± 1284	907 ± 841
		p = 0.009‡	p = 0.619‡
GKO	**(n= 36)**	1514 ± 720	853 ± 765

*Data from N. Goes are included in the summary
**Experiments were age and sex-matched
^Wild type mice are littermates or otherwise notes
‡The comparisons between sex and age matched wild type and GKO mice were performed using a two way Student's t test (N. Goes).

VII. FIGURES

Figure 2.1: Photomicrographs of IIP staining for MHC class I in kidneys from wild-type and GKO mice in the basal state. Class I in wild-type (top) shows staining of arterial endothelium (arrow) and GKO mice (bottom) have an absence of staining. The photomicrographs were taken at the same magnification (160 X).



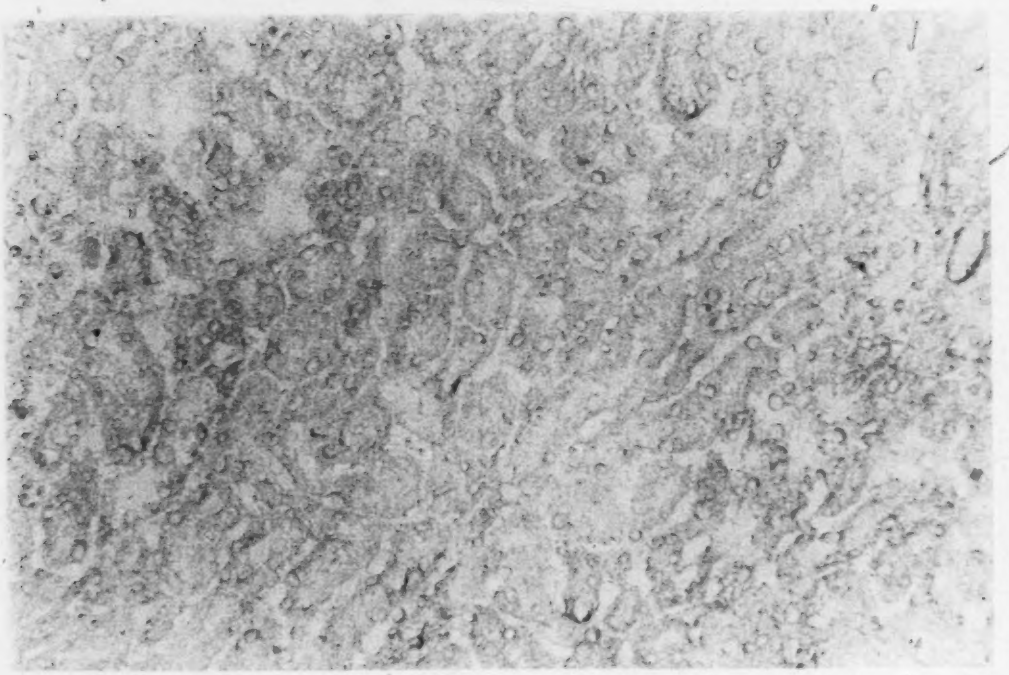
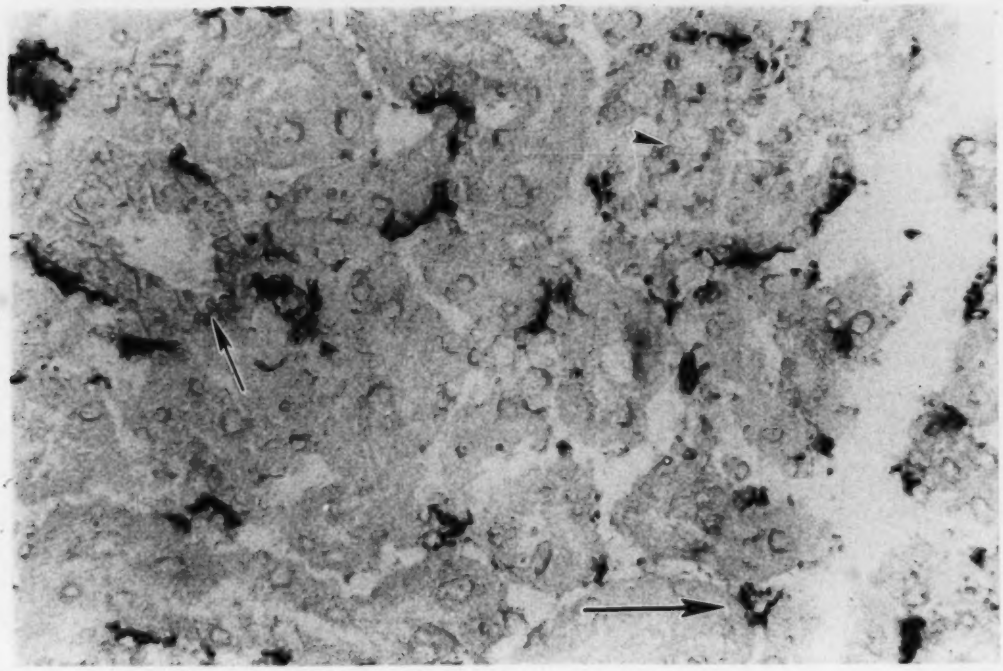


Figure 2.2: Photomicrographs showing IIP staining for MHC class II in kidneys from wild-type and GKO mice in the basal state. Wild-type mice (top) showed staining of interstitial cells (large arrow) and GKO mice (bottom) have similar staining patterns. The arterial endothelium is not stained (small arrow). The photomicrographs were taken at the same magnification (250 X).



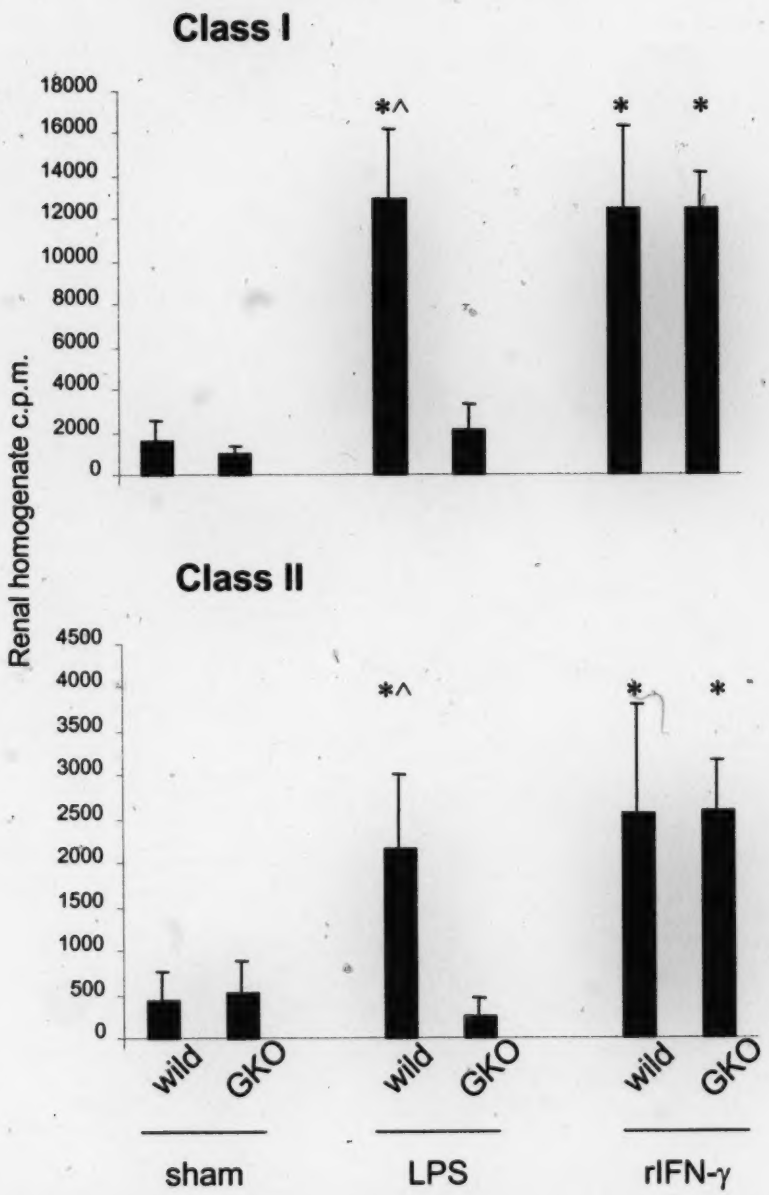


Figure 2.3: MHC class I and II expression in kidney homogenates (RABA) after LPS and rIFN-γ administration. LPS (25 μg) or rIFN-γ (100,000 I.U.) was injected i.p. on day 0 and the mice were harvested on day 3. *Significant differences between the sham and the LPS or rIFN-γ groups, or ^ between the wild-type and GKO mice with the same treatment. The comparisons were performed using ANOVA, $p < 0.05$.

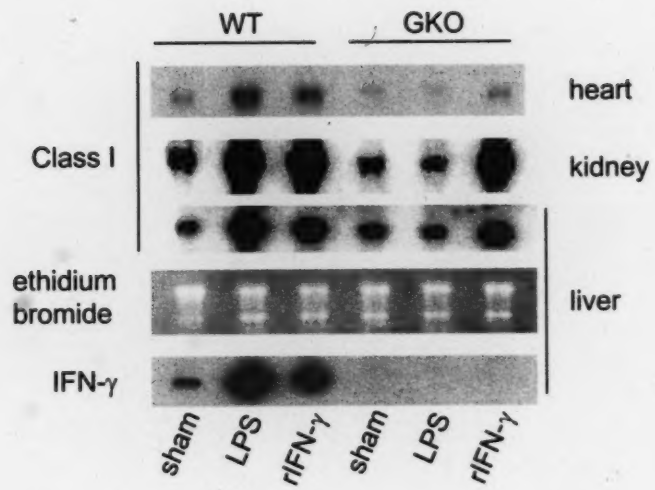
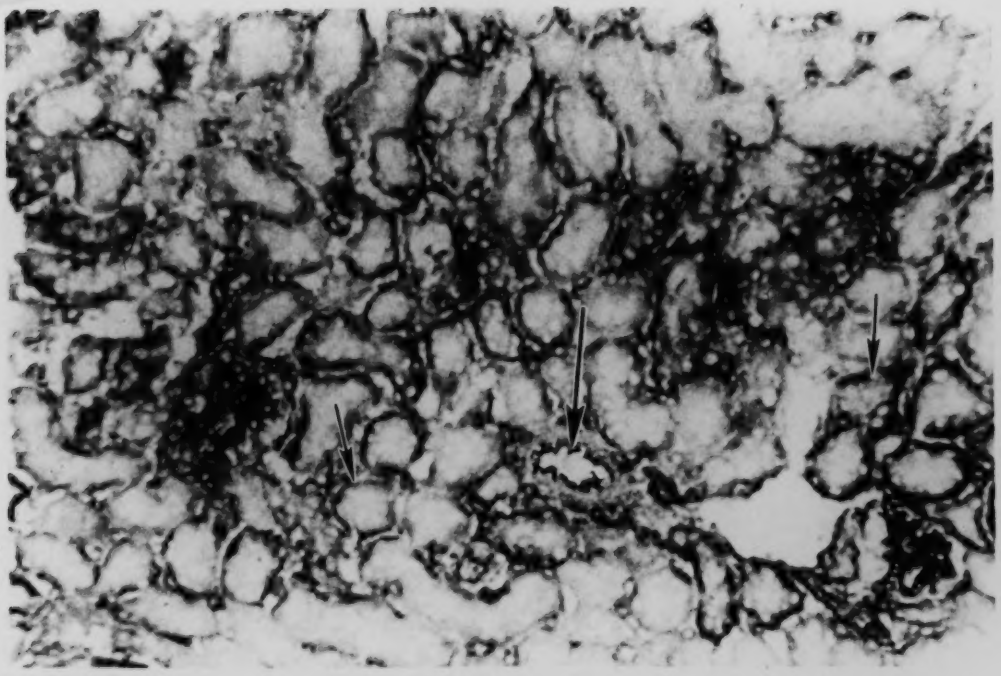


Figure 2.4: MHC class I mRNA expression after LPS and rIFN-γ administration. LPS (25 μg) or rIFN-γ (100,000 I.U.) was injected i.p. on day 0 and the mice were harvested on day 3. Class I was measured by northern blot. Ethidium bromide is shown to monitor total RNA quality. IFN-γ was amplified by RT-PCR.

Figure 2.5: Photomicrographs of IIP staining for MHC class I in kidneys from wild-type and GKO mice after LPS injection. Wild-type mice (top) had staining of the arterial endothelium (large arrow) while GKO mice (bottom) showed an absence of staining. Tubular staining is evident (small arrow) in wild-type mice (top) only. The photomicrographs were taken at the same magnification (160 X).



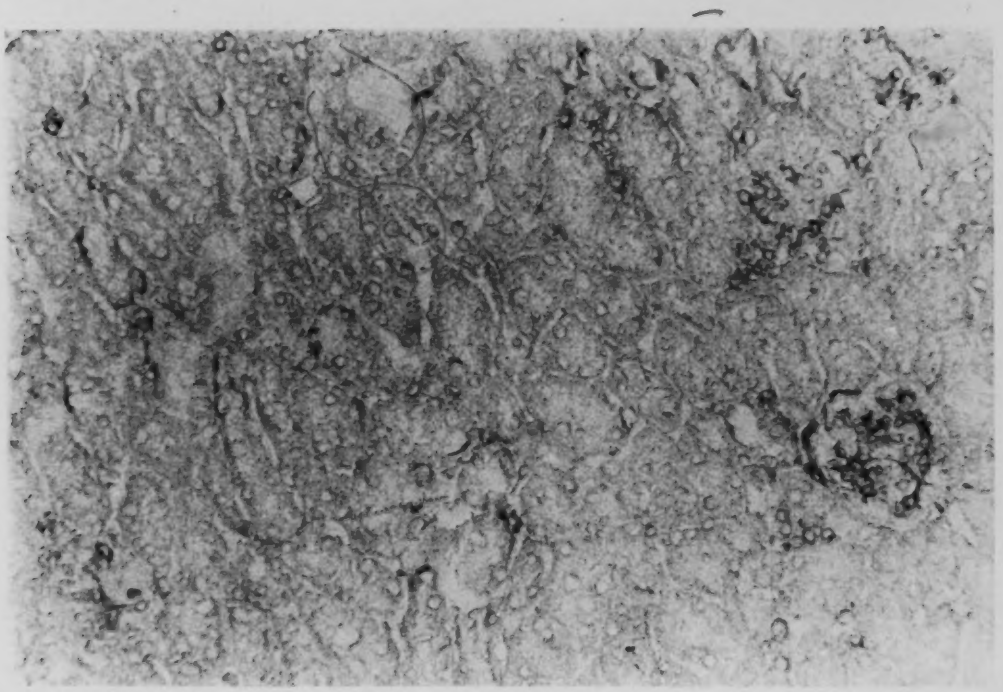
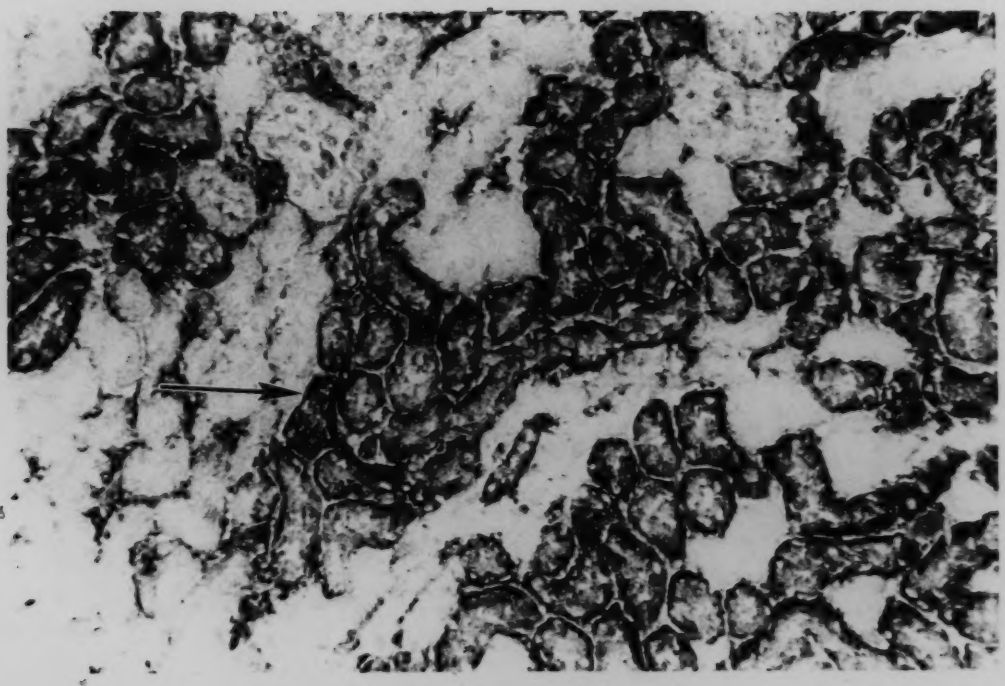


Figure 2.6: Photomicrographs of IIP staining for MHC class II in kidneys from wild-type and GKO mice after LPS injection. Wild-type mice (top) had staining of the tubular epithelium (large arrow) while GKO mice (bottom) showed an absence of staining. Interstitial cell staining is evident (small arrow) in the GKO mice (bottom). The photomicrographs were taken at the same magnification (160 X).



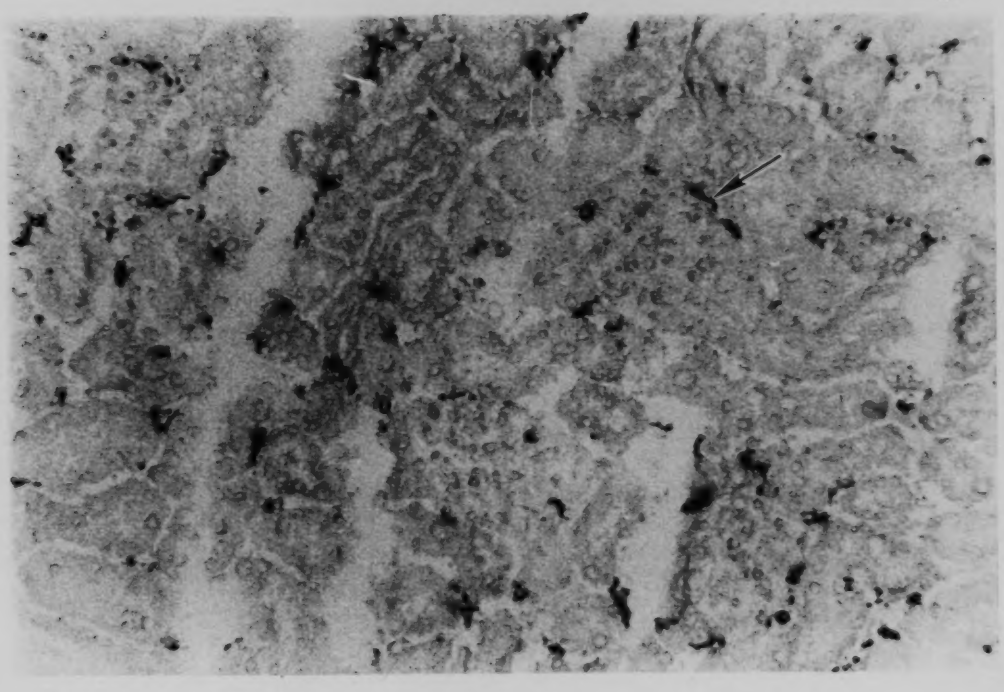
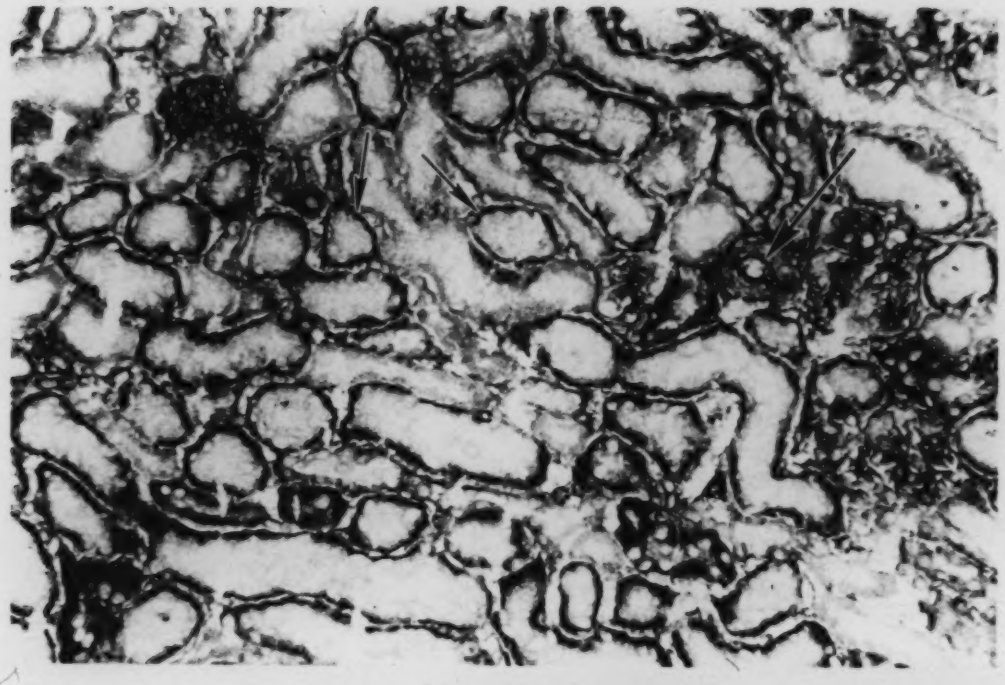


Figure 2.7: Photomicrographs showing IIP staining for MHC class I in kidneys from wild-type and GKO mice after rIFN-γ injection. Staining is evident on the tubular epithelium in both wild type (top) and GKO (bottom) mice (small arrow). The artery in both types of mice are positive for class I (large arrow). The photomicrographs were taken at the same magnification (160 X).



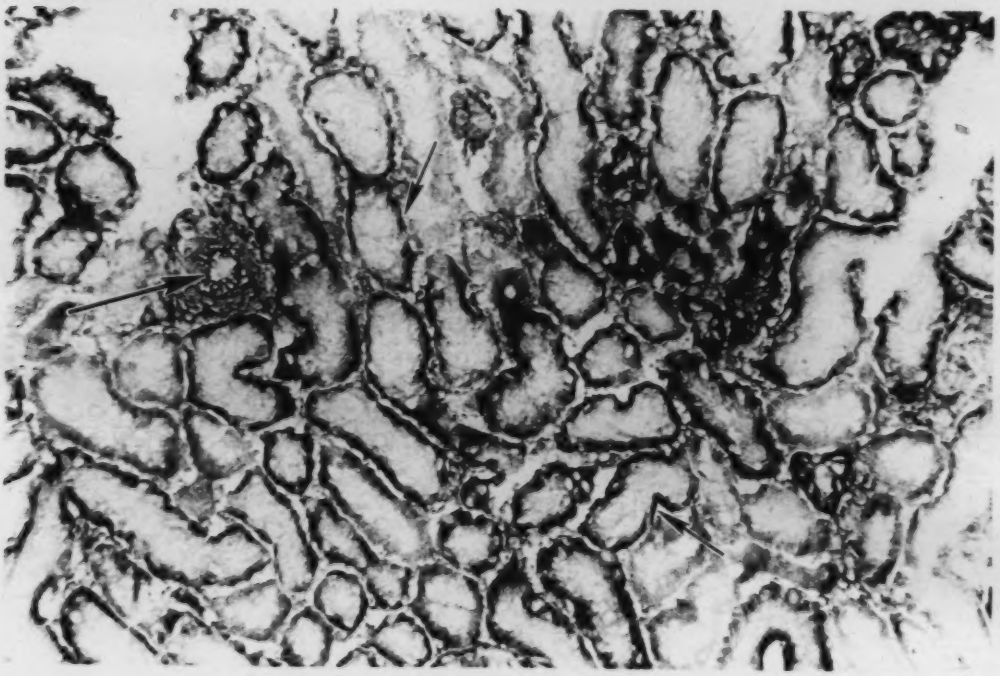
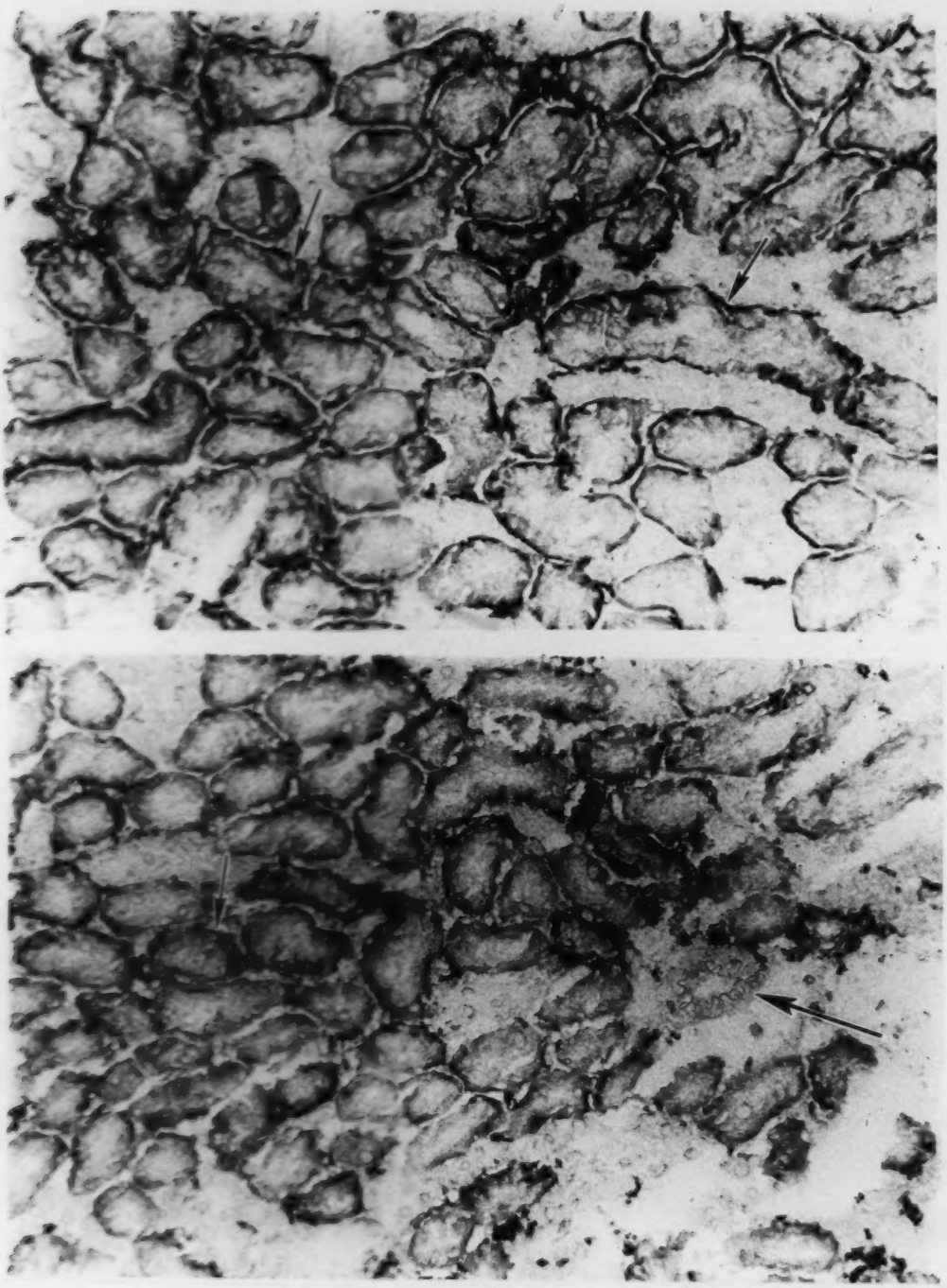


Figure 2.8: Photomicrographs showing IIP staining for MHC class II in kidneys from wild-type and GKO mice after rIFN-γ injection. Staining is evident on the tubular epithelium in both wild type (top) and GKO (bottom) mice (small arrow). The artery in both types of mice remains negative for class II (large arrow). The photomicrographs were taken at the same magnification (160 X).



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CHAPTER 3

Regulation of Antigen Presentation Genes *In vivo*

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I. PREFACE

MHC class I and II are increased not only in response to LPS and IFN-γ but also in response to local injury and allograft rejection. The antigen presenting machinery had never been studied in the kidney, so I investigated the regulation of several of the antigen presenting genes for class I and II under conditions known to increase MHC levels in the kidney.

II. INTRODUCTION

It is unknown if renal cells present immunogenic antigen. Induced MHC molecule expression in a graft is associated with poor initial function and reduced graft survival (1;2), while lack of MHC expression has been correlated with increased graft survival (3;4). Previous studies showed that class I and class II

product closely correlates with their respective levels of mRNA (5). IFN-γ induces expression of the MHC family of genes including class I, TAP, LMP class II, Ii and H2-M along with the class II transactivator (*CIITA)* (6). The full induction of *CIITA, class II* and *class I* genes requires the IFN-γ -inducible transcription factor IFN regulatory factor 1 (IRF-1) (7;8).

I explored the regulatory mechanisms of several antigen presentation gene mRNA levels in the mouse kidney. Specifically, I investigated the roles for IFN-γ and the transcription factor IRF-1 on the steady-state mRNA levels of H2-Mα, TAP1, and LMP7. I demonstrate that basal expression of these genes is independent of IFN-γ and that IRF-1 is critical for their induction in response to systemic stimulation. Furthermore, H2Mα, TAP1 and LMP7 mRNAs are induced in response to acute ischemic injury in WT kidney. These results correlate with published results generated in this laboratory on class I and class II. My results suggest that the kidney has a functioning pathway for presenting peptides in the grooves of class I and class II molecules and that the induction of the pathway is at least partially dependent on the transcription factor IRF-1.

III. MATERIALS AND METHODS

Wild-type mice. BALB/c mice aged 6-12 weeks were obtained from Jackson Laboratories, Bar Harbor, ME (BALB/cByJ), from Charles River Laboratories, Montreal, Quebec (BALB/cAnNCrlbr), or from the Health Sciences Laboratory Animal Services at the University of Alberta (BALB/ccCr/AltBM). 129/SvJ mice

and BALB/cJxA/J F_1 (CAF_1/J) aged 6-12 weeks were obtained from Jackson Laboratories, Bar Harbor, ME. The Health Sciences Laboratory Animal Services at the University of Alberta sustained all mice. All experimental procedures were in agreement with animal care protocols enforced by the institution review board.

IFN-γ gene-disrupted (GKO) mice (9). (same as in chapter two) BALB/c heterozygous for the IFN-γ gene and BALB/c mice with intact wild-type IFN-γ genes were provided to us as a gift from Dr. Tim Stewart (Genentech Inc., South San Francisco, CA). BALB/c, GKO and wild-type homozygous mice were genotyped by PCR amplification of tail skin DNA as described (5;9).

IRF-1 gene-disrupted (IRF-1 KO) mice. IRF-1 KO mice were provided by Dr. Tak Mak (Ontario Cancer Institute, Toronto, ON). Homozygosity of all IRF-1 KO mice was confirmed by testing all experimental mice with a RT-PCR system for absence of wild-type IRF-1 mRNA.

Local renal ischemic Injury. Mice 8-12 weeks of age were anaesthetised with 2,2,2-tribromomethanol in tert-butyl alcohol (Avertin) by intraperitoneal (i.p.) injection. The left renal pedicle was identified through a midline section and occluded with a micro-bulldog clamp for 60 minutes. Before closure the kidney was inspected to ensure reperfusion and the abdominal cavity was filled with warm saline. Sham -operated control mice underwent a simple laparotomy under identical conditions. The mice were killed at 1, 5, 7, 14, or 21 days.

P815 tumor allografts. The P815 mastocytoma tumor ($H2^d$, American Type Culture Collection, Rockville, MD) was passaged in DBA/2 mice from Jackson Laboratories (Bar Harbor, ME). The ascites fluid was removed and 15-20 X 10^6 cells were injected i.p. into mice. The mice were killed after one week and tissues were harvested by snap freezing in liquid nitrogen. As previously described (10), mice reject the allogeneic P815 (or P815 differing on minor H) mastocytoma ascites tumour cells.

R4-6A2 (anti IFN-γ mAb) was ammonium sulphate precipitated and then put through a DE52 anion exchanger column (Whatman, Hillsboro, OR), and concentrated by Amicon ultrafiltration. The protein concentration was determined by a modified Lowry method, adjusted to 1 mg/ml and kept frozen at -70°C. Radioiodination was performed by Iodogen method (Pierce Chemical Co., Rockford, IL) (11). Anti IFN-γ mAb (R4-6A2) (12) was injected i.p. (100 μg) for 7 days, starting one day before the injection of P815 cells as described elsewhere (10).

Recombinant IFN-γ injections (same as in chapter two). BALB/c mice were injected i.p. on day 0 with 100 000 U of rIFN-γ, (gift from Dr. Tim Stewart (Genentech Inc., South San Francisco, CA).) and were sacrificed at various time points.

Northern blot analysis. Total RNA was extracted from one to three mice according to a modification of the method described by Chirgwin *et al* (13). Tissues were homogenised with a polytron in 4 M guanidinium isothiocyanate and the RNA was pelleted through a 5.7 M $CsCl_2$ cushion (14). The 260/280 RNA concentrations were taken and equal amounts from 5-10 mice were pooled for electrophoresis. Northern blots were prepared by using 10 to 20 μg of total RNA electrophoresed through a 1.5% agarose gel in the presence of 2.2 M formaldehyde, with transfer to nitrocellulose filters. Blots were then hybridised with a ^{32}P-labelled cDNA probe for class I (HLA-A3), class II (I-Aα) followed by exposure to Kodak X-Omat AR film at -70°C with an intensifying screen. Loading and quality of RNA was monitored by probing the filter for β-actin (Northerns done by A. Pahl or V. Ramassar).

RT-PCR. Total RNA from 5-10 mice per group was reverse transcribed into cDNA using Superscript reverse transcriptase (BRL, Burlington, Ontario) at 42°C for 45 minutes. PCR amplification of cDNA was performed in a Perkin Elmer Cetus thermal cycler using Taq DNA polymerase and specific oligonucleotide primers for H-2Mα, LMP7, TAP1 or HPRT (control), completed with 25 to 28 cycles with an annealing temperature of 55 ° C. The RT-PCR products were southern blotted and probed with ^{32}P-labelled cDNA (Klenow enzyme, Roche, Laval, QC) or oligonucleotide (PNK enzyme, BRL, Burlington, ON) probes. Primers for H-2Mα, LMP7 and TAP1 were chosen utilising OLIGO 4.0 (National Biosciences, Plymouth, MN) based upon sequences obtained from Genebank.

The IRF-1, IFN-γ and CIITA primers and probes have been previously reported (7;15).

The H-2Mα sense primer (5' TCTCCCAGAACACCAGAGTG) and the antisense primer (5'GCGGTCAATCTCGTGTGTCA) amplified a fragment of 393 basepairs (bp), detected by southern blot with a specific internal antisense probe (5'TGGCTCCCTCCACAGGGGCTGAGTGAAGCTGCC). The LMP7 sense primer (5'ATTAACCCTTACCTGCTTGG) and antisense primer (5' GTTGTCTCTGTGGGTAGCAT) amplified a 396 bp fragment, detected by southern blot using an internal oligonucleotide probe: 5' CCGGTAACCACTGTCCATCACCCCGTAGG 3'. A 556 bp fragment of TAP1 cDNA was detected in the same manner using a TAP1 sense primer (5' AACCAGCCCAAAGTCCAGG), antisense primer (5' AGAAGAACCGT CCGAGAAGCC) and an internal oligonucleotide probe: 5'GCTCGGGCCAAGGCCACTGCCTGTCGTCGC. The PCR primers and probe for IFN-γ were described previously (5). HPRT was amplified using specific primers: sense (5' GTTGGATACAGGCCAGACTTTGTTG), antisense (5' GAGGGTAGGCTGGCCTATGGCT) and an internal specific probe (5' GCAGGTGTTGAGTCCTGTGGCCATCTGCCCTAG). HPRT was used as a loading control in each experiment. Only one HPRT blot per figure is shown for space consideration.

IV. RESULTS

Recombinant IFN-γ induces the genes for antigen presentation *in vivo*. Systemic IFN-γ induces MHC in remote organs such as the kidney (5;7). However, it is not known if IFN-γ regulates specific components of the antigen presentation machinery in the kidney. I treated wild-type mice with rIFN-γ and harvested the kidneys at several time points (Figure 3.1). By RT-PCR, LMP7 and TAP1 mRNAs were induced markedly early in the time course (Figure 3.1A). As controls, IRF-1 mRNA was induced early and class I mRNA levels were increased later (7). In Figure 3.1B, class II (control) and H-2Mα were induced late, peaking at 24 hours and slowly declining to baseline by day 7. The CIITA mRNA was induced early in the time course (16), as expected, since CIITA is the transcriptional transactivator for the class II family of genes. Thus, components of the antigen presentation machinery in the kidney are regulated with different but predictable kinetics in response to systemic rIFN-γ.

IFN-γ may be self-amplifying, through induction of its own expression (17). I administered rIFN-γ to GKO and WT mice to see the effect of endogenous IFN-γ on the response to exogenous IFN-γ (Figure 3.2). IFN-γ mRNA was undetectable in GKO mice with the primers used (5), with or without rIFN-γ administration. However, the GKO mice responded to exogenous IFN-γ with an increase in H-2Mα, LMP7 and TAP1 mRNA levels (Figure 3.2A). Therefore, as with class I

and II (Chapter 2), endogenous IFN-γ production was not essential for the induction of H-2Mα, LMP7 and TAP1 mRNA in response to exogenous rIFN-γ .

Impaired induction of the antigen presenting genes in kidneys of IRF-KO mice after induction by rIFN-γ. IRF-1plays an important role in the regulation of class I and class II *in vivo* (7;18). IRF-1 is induced by IFN-γ through STAT 1α. Since IRF-1 has been shown to regulate CIITA, LMP2 and TAP1 (7;19;20) I investigated the role of IRF-1 in regulating other members of the antigen presentation machinery in kidney *in vivo*. IRF-1 KO and WT (129/SvJ) mice were injected with rIFN-γ and kidneys were harvested at 72 hours. TAP1, LMP7, and class I mRNAs were induced in the wild-type kidneys. The induction of the mRNA was markedly reduced in the kidneys from IRF-1 KO mice (Figure 3.2B), as seen previously for MHC class I protein and CIITA mRNA levels (7). These results indicate that IRF-1 is involved in the regulation of TAP1 and LMP7 in response to IFN-γ in kidney *in vivo*.

Induction of antigen presenting genes in response to systemic stimuli is dependent on IFN-γ and IRF-1. Systemic inflammatory stimuli such as contact sensitivity or exposure to endotoxin cause a cytokine response. MHC expression is induced in both cases by the endogenous release of IFN-γ (5;7;10). In response to LPS, wild type mice show a strong induction of the antgen presenting machinery mRNA levels in kidney homogenate at day 4 (Figure 3.3A). There is very little, if any, induction of H-2Mα, TAP1 or LMP7 in the GKO mice or

of H-2Mα in the IRF-1KO mice (Figure 3.3B). Class II mRNA induction in response to LPS at day 4 is also reduced in the IRF-1KO mice compared to the wild type mice, similar to earlier studies (7). Thus the induction of the antigen presenting machinery in kidney in response to LPS is dependent on the IFN-γ pathway.

H2-Mα, LMP7 and TAP1 mRNA are induced by allogeneic stimulation *in vivo* and the induction is blocked by anti-IFN-γ monoclonal antibodies or gene-disruption. BALB/c mice rejecting DBA/2 P815 ascites tumours release endogenous IFN-γ systemically, induce class I and class II molecule expression in many organs and induce IFN-γ mRNA the kidney (10;17). In response to P815, MHC class I and II molecules are upregulated at day 7 and this induction is dependent on IFN-γ and IRF-1 (5;7). I hypothesised that the regulation of the antigen presentation machinery in the kidney in response to P815 was also dependent on IFN-γ. By RT-PCR, I examined LMP7, TAP1, H-2Mα, and CIITA mRNA levels at day 7 after P815 injection (Figure 3.4). In wild-type mice, TAP1, LMP7, H-2Mα, CIITA, and IFN-γ, mRNAs were strongly induced by P815 (~3-10 fold). The induction was lost with both anti-IFN-γ antibody treatment and in the mice lacking IFN-γ (GKO). These results exactly parallel the patterns of induction in response to P815 that we have seen for class I and II protein expression (5). Similarly, H2-Mα, LMP7 and class I mRNA expression in IRF-1 KO mice was reduced compared to the wild-type mice after P815 stimulation (Figure 3.5).

Thus the induction of the antigen-presentation machinery following allogeneic stimulation is dependent on IFN-γ and on IRF-1 production.

Non-specific ischemic injury induces the antigen presentation machinery. MHC class I and II molecules are induced approximately 2- to 3-fold in response to both ischemic renal injury and toxic renal injury (5;8;21). I examined the injured kidneys of mice after cross clamping to induce ischemic injury in the kidney tubules (5). We cross clamped the left renal pedical to injure the kidney and measured the expression of H-2Mα, TAP1, LMP7 and class I mRNA at day 7, 14 and 21. LMP7 mRNA and class I (22) levels reached maximum expression early in the injured kidney at day 7 continuing to day 14, while TAP1 and H2Mα mRNA levels and class II (22) peaked between 7 and 21 days after injury (Figure 3.6A). The right kidneys of these mice were similar to the sham treated controls, indicating that the observed changes were not due to a systemic response. In the GKO mice at day 7, H-2Mα, LMP7 and TAP1 mRNA levels were induced in the injured left kidney compared to the sham operated mice (Figure 3.6B). The induction in GKO mice was reduced compared to the wild-type mice. Thus non-specific injury induces the antigen presentation machinery *in vivo* and the induction is partially dependent on IFN-γ.

V. DISCUSSION

The present experiments demonstrate that both the exogenous and endogenous pathways of antigen presentation are rapidly responsive in the kidney to systemic and local stimuli. H-2Mα, TAP1 and LMP7 mRNAs are present at low levels in the basal state and are upregulated in response to exogenous rIFN–γ, inflammatory stimuli, and non-specific local renal injury. Basal expression of H-2Mα, TAP1 and LMP7 mRNA was not dependent on IFN-γ or on IRF-1. The response to inflammatory stimuli was dependent on IFN-γ and on the transcription factor IRF-1. In response to renal injury H-2Mα, LMP7 and TAP1 mRNA expression were induced. Thus the induction of the renal antigen processing machinery is an early feature of many responses in which renal MHC expression is also induced.

H-2Mα and MHC class II are regulated in similar patterns in response to systemic and local stimuli. H-2Mα is increased here in response to rIFN-γ, P815 and ischemic injury, parallel to our earlier studies on class I and II in the kidney (5). In response to rIFN-γ, CIITA (and thus the class II family) is regulated by IRF-1 (7). Furthermore, as with the earlier class I and II studies, H-2Mα mRNA expression is almost completely IFN-γ-dependent in response to systemic stimuli but is only partially IFN-γ dependent in response to local injury. Thus there is a portion of H-2Mα induction in response to injury that is partially IFN-γ independent. The promoters for HLA-DMA, the human homologue to H-2Mα, and DMB have been described (24). They share similar architecture to the class II family of promoters and are regulated by CIITA and the RFX transcription factors

(24;25). From our data with rIFN-γ in wild-type mice and with P815 in GKO and IRF-1 KO mice, H-2Mα mRNA levels are also regulated by IFN-γ and IRF-1 in the kidney, likely via CIITA.

TAP1 and LMP7 mRNA and class I are regulated by the same stimuli in the kidney. In response to systemic rIFN-γ and P815, the antigen presenting machinery is induced. Of interest, in the IFN-γ time course, the steady state TAP1 and LMP7 transcripts were upregulated early (by 2 hours) compared to class I transcripts, which peaked at 24 hours. The IFN-γ induction of TAP1 and class I is partially dependent on IRF-1, in concordance with earlier studies on class I and II (7). After P815 injection, class I is massively induced at both the mRNA and protein levels by day 7, corresponding to increases in class I staining on the tubular epithelium by immunohistochemistry (5;18). In the GKO and IRF-1 KO mice, this staining is almost completely lost (5;18). Similarly, TAP1 and/or LMP7 mRNA induction is reduced in the GKO mice, in wild-type mice treated with neutralising anti-IFN-γ antibodies, or in IRF-1KO mice. Thus, like class I, TAP1 and LMP7 mRNA levels are induced by IFN-γ-dependent mechanisms in response to systemic stimuli.

The early induction of TAP1 and LMP7 by IFN-γ may reflect a fundamental difference between the promoters of these genes and the regulatory elements of genes with typical class I promoter organisation. Several regulatory elements have been identified within the promoter of class I including regions I and II of Enhancer A and the interferon stimulated response element (ISRE) (26) which together make the class I regulatory complex. While NF-κB is though to bind

region I (27), IRF- 1 and other IRF family members *in vitro* bind the ISRE (28). Conversely, enhancer A is absent in the bi-directional promoter of TAP1 and LMP2. The nature of class I gene expression may therefore be attributed to proteins which bind the class I regulatory complex. The IFN-γ activated site (GAS) confers rapid responses to IFN-γ and the IRF-1 transcription factor has a GAS in its promoter region (29;30). IRF-1-sensitive genes are therefore responsive to the second wave of activation through IFN-γ. This could explain the rapid *in vivo* induction of TAP1 in response to rIFN-γ as the result of proteins binding at the GAS. The later induction of class I, in which the GAS element is absent, could be explained as a function of IRF-1 binding its response element (7;19). The similar expression patterns of LMP7 and TAP1 mRNAs suggest that they may be regulated by similar genetic elements. Our homology search for enhancer elements within the LMP7 gene identified a potential ISRE and a GAS located upstream and downstream, respectively, of the transcriptional start –site. The LMP7 promoter has not been fully characterised, leaving open the question of its regulation by IFN-γ and IRF-1. IFN-γ induces TAP1 and CIITA expression through activation of STAT1α binding the GAS in target promoters (29). IRF-1 may also be involved in the regulation of the antigen presenting machinery in kidney *in vivo* since IFN-γ upregulates TAP1 and CIITA mRNA and class I expression through IRF-1

Our injury model demonstrates that the antigen presenting machinery is induced by a complex local stimulus such as renal ischemic injury. The injury response is stereotyped and independent of the inducing stimulus (31). The

induction of class I and II utilises IFN-γ- and IRF-1-dependent mechanisms as well as pathways independent of these factors (5;8). Looking at ischemic injury, TAP1 and LMP7 are induced by day one (not shown), and remain high until day 7 and return to baseline at day 14. These differential patterns of induction may illustrate the various levels of influence playing on each gene. For example, H-2Mα and class I are induced late in the response to rIFN-γ (Figure 3.1) perhaps reflecting their requirements for late transcriptional activators such as CIITA for H-2Mα. Furthermore, during the injury response, CIITA is inducible in the absence of IFN-γ (8) availing it for activation of target genes such as H-2Mα in the GKO mice. The MAP kinase stress response pathways have been implicated in an ischemic heart model, but the signals involved in the IFN-γ-independent MHC induction after injury remain to be elucidated. From our data, the antigen presenting machinery is induced in response to injury and this response is only partially dependent on IFN-γ.

It is unknown whether the kidney presents antigen. We have shown that the genes responsible for antigen presentation are induced in the kidney and that this induction correlates with MHC induction. I demonstrated the necessity for IRF-1 in the induction by IFN-γ of H-2Mα, TAP1 and LMP7 mRNAs. The availability of endogenous peptides limits expression of class I in some cells (32). It is possible that the decreased class I product expression observed in IRF-KO and GKO mice (5;7) reflects deficiencies in the expression of the antigen presentation apparatus as we have shown here. Overall the demonstration that

stimuli regulate TAP1, LMP7 and H-2Mα argues that the MHC induced inkidney is relavent and loaded with peptide.

VI. FIGURES

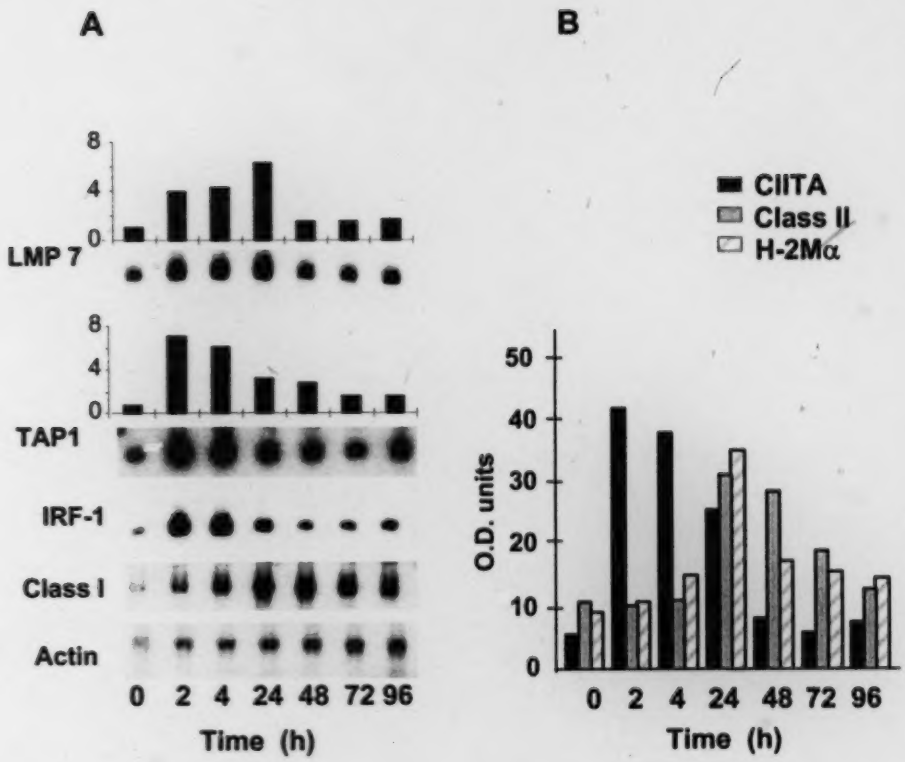

Figure 3.1: Time course of rIFN-γ induction the antigen presenting machinery in mouse kidney. CAF_1/J mice were injected i.p. with 100,000 U. of rIFN-γ and kidneys were harvested at 2, 4, 24, 48, 72, and 96 hours after injection. (A) RT-PCR was done for LMP7 and TAP1, and IRF-1 using standard conditions, Southern blotted and probed. Class I was detected by northern blot and probed with class I cDNA (controls contributed by M. Hobart). Actin was used as a loading control on the northern blot. (B) RT-PCR was performed for CIITA and H-2Mα and detected using cDNA and oligonucleotide probes, respectively. Class II was detected by northern blotting and probed with class II cDNA (CIITA and class II contributed by V. Ramassar). Units are shown as arbitrary densitometry units.

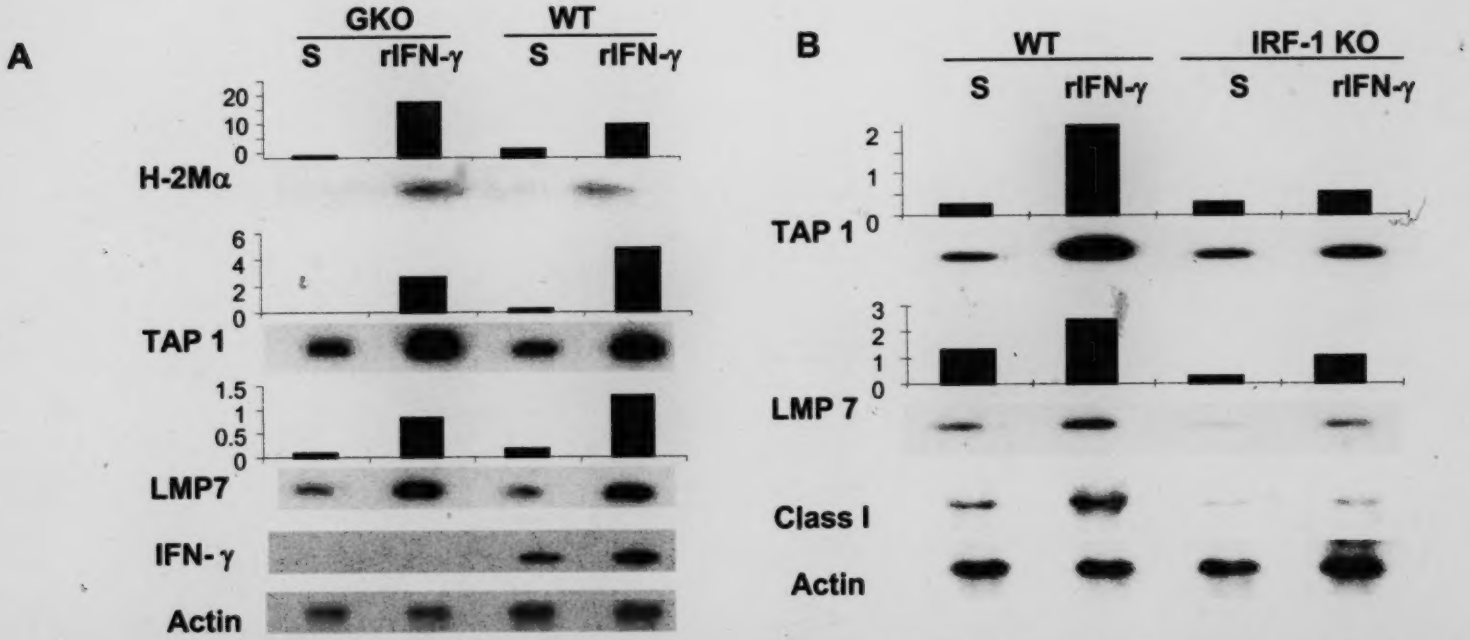

Figure 3.2. Effect of systemic stimulation by rIFN-γ on the antigen presentation machinery. Mice were injected with 100 000 U of rIFN–γ and their kidneys were harvested at 72 hours.(A) BALB/c (WT) or GKO mice were injected with saline (S) or rIFN-γ. RT-PCR was done for H-2Mα, TAP1, and LMP7, Southern blotted and probed. HPRT was used as a loading control (not shown). Y-axes are expressed as arbitrary Phosphor Imaging units. (B) (panel contributed by A.. Pahl and V. Ramassar) 129/SvJ (WT) or IRF-1KO mice were injected with saline (S) or of rIFN-γ. RT-PCR was done for TAP1 and LMP7 using standard conditions, Southern blotted and probed. Class I was detected by northern blot. Actin was used as a loading control on the northern blot. Y-axes are expressed as arbitrary densitometry units.

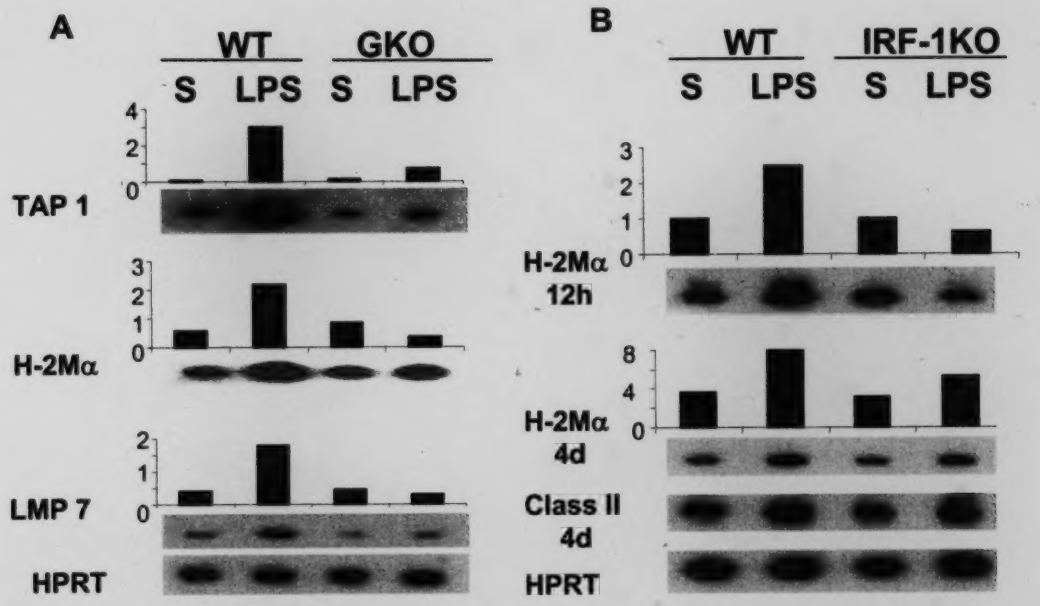

Figure 3.3: Effect of systemic stimulation by LPS on the antigen presentation machinery. Mice were injected with 25μg of LPS and their kidneys were harvested at various times.(A) BALB/c (WT) or GKO mice were injected with saline (S) or LPS and harvested on day 4. RT-PCR was done for H-2Mα, TAP1, and LMP7, Southern blotted and probed. Y-axes are expressed as arbitrary Phosphor Imaging units. (B) 129/SvJ (WT) or IRF-1KO mice were injected with saline (S) or LPS. RT-PCR was done for H-2Mα, Southern blotted and probed. Northern blot was used to quantitate class II. Y-axes are expressed as arbitrary Phosphor Imaging units.

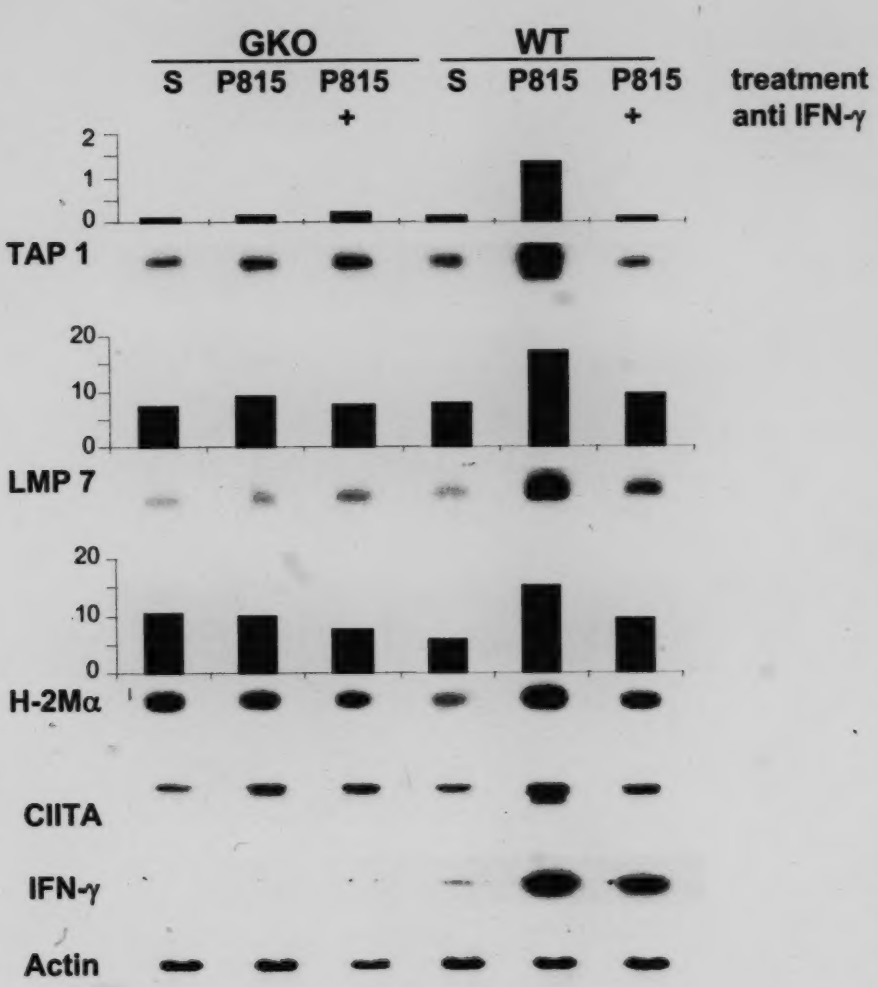

Figure 3.4: P815 allostimulation induces the antigen presentation machinery. BALB/c (WT) and GKO mice were injected with P815 cells on day 0 and were harvested at day 7. Anti IFN-γ was injected i.p. (100 μg) for 7 days, starting day minus one. RT-PCR amplification of TAP1, LMP7, H-2Mα, and CIITA mRNA (28 cycles) and IFN-γ mRNA (30 cycles) was performed, Southern blotted and probed. Actin, CIITA and IFN-γ blots done by V. Ramassar. Quantitation was performed by densitometry and is expressed as arbitrary units.

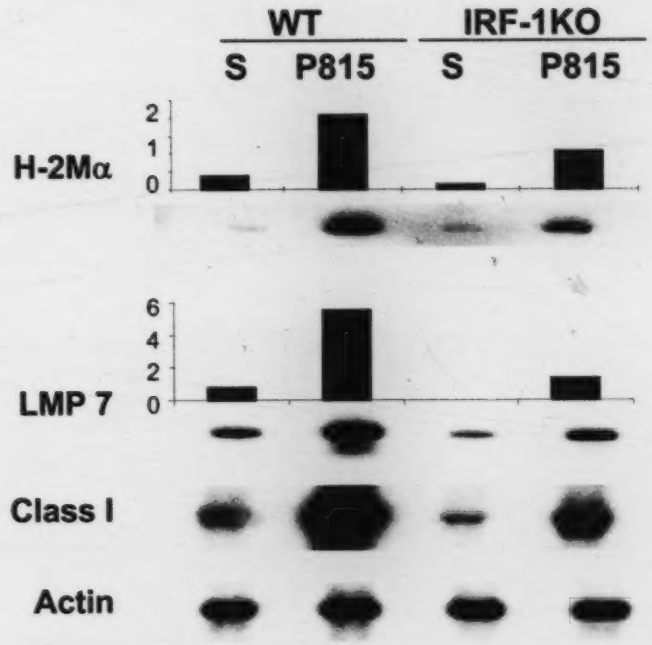

Figure 3.5: P815 allostimulation induces the antigen presentation machinery in IRF-1KO mice at reduced levels. 129/SvJ (WT) and IRF-1KO mice were injected with P815 cells on day 0 and were harvested at day 7. RT-PCR amplification of H-2Mα and LMP7 mRNA (28 cycles) was performed, Southern blotted and probed. Class I and actin were detected by northern blotting (V. Ramassar). Y-axes depict arbitrary Phosphor Imaging units of which mRNA loading was corrected by HPRT (not shown).

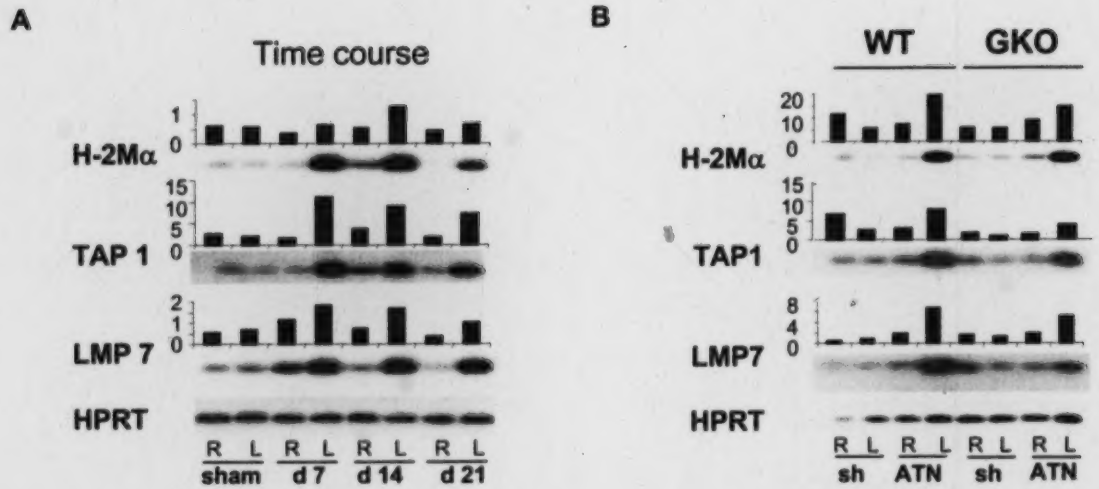

Figure 3.6: Ischemic renal injury induces the antigen presenting machinery.(A)Time course of H2-Mα, TAP1 and LMP7 after ischemic injury in BALB/c mice. H2-Mα, TAP1, and LMP7 mRNA were detected by RT-PCR, Southern blotted and probed. HPRT was used as a loading control. The Y-axes depict arbitrary Phosphor Imaging units (B) BALB/c (WT) and GKO mice were injured by ischemia (ATN) to the left (L) kidney and harvested at day 7. The right (R) kidney serves as the contralateral control. H-2Mα, TAP1, and LMP7 mRNA were detected by RT-PCR, Southern blotted and probed. HPRT was used to monitor RNA. Sham operated mice (sh). The Y-axes depict arbitrary Phosphor Imaging units.

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CHAPTER 4

Mouse Class II Transactivator: cDNA Sequence and Amino Acid Comparison With The Human Class II Transactivator

Chapter 4

Mouse Class II Transactivator: cDNA Sequence and Amino Acid Comparison With The Human Class II Transactivator

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I. PREFACE

CIITA had been shown to be required for class II expression in humans, and reagents against the human CIITA were used to identify the gene and molecule in the mouse. The human cDNA was available but the genomic clone was unknown. I thus set out to clone the cDNA for mouse CIITA and therefore have a probe to find the full-length genomic clone.

II. INTRODUCTION

The human CIITA cDNA is 4543 basepairs (bp) in length (1) with an open reading frame of 3390 bp, coding for a predicted protein of 1130 amino acids (aa). The carboxy-terminal region of CIITA is thought to be responsible for class II transcription specificity, while the amino-terminal acidic region likely binds non-specific transcription factors (2;3). If these regions are important for MHC class II

induction, it is hypothesised that they will be highly conserved in mouse and man, but the full-length mouse cDNA sequence had yet to be described.

III. MATERIALS AND METHODS

In order to clone a full length cDNA for mouse CIITA, a ~400 bp sub-fragment of the mouse cDNA (bp 4-422 of GenBank #U46562) was first generated by RT-PCR using total cellular RNA from BALB/c spleen and PCR primers based on the human cDNA sequence (4). The resulting 419 bp fragment was cloned into pBluescript SK- and confirmed to be correct by DNA sequencing using fluorescently labelled dideoxy terminators (Perkin-Elmer Applied Biosystems Division, Foster City, CA). The cloned insert was excised from a 0.8% LMP agarose gel and labelled directly with P^{32}-αdCTP using random hexamers and Klenow enzyme. The labelled probe was used to screen a λ-ZAP splenic cDNA library generated from a NOD/alt mouse, and from the approximately 4 x 10^5 plaques screened, 13 positive clones were obtained. Seven of these clones were plaque purified, subjected to *in vivo* subcloning into pBluescriptSK- using helper phage R408 (Stratagene, San Diego, CA.), and mapped by restriction enzyme digestion and agarose gel electrophoresis. cDNA insert sizes ranged from approximately 400-5200 bp, with no inserts being the same size. The longest insert (clone mCIITA-7) was sequenced on both strands using fluorescently labelled dideoxy terminators and a primer walking strategy. Sequence analysis was performed using the DNA Strider 1.2 program. The sequence was compared with the published human CIITA sequence (GenBank

#X74301) and similarity and secondary structure analyses were performed at the Alberta Peptide Institute (API) using the PPSP and SEQSEE programs (5).

IV. RESULTS AND DISCUSSION

The clone mCIITA-7 contains a 5178 bp insert (DNA sequence not shown; GenBank accession #U60653) which contains a single open reading frame of 3236 bp, encoding a protein of 1078 amino acids. Alternatively spliced human CIITA mutants with no known function have been described by Riley et al. (1995). It remains to be determined if any of the mouse clones 1-6 are alternatively spliced forms of mouse CIITA-7. Mouse CIITA-7 is compared to the deduced human CIITA protein in Figure 4.1, which has an open reading frame of 1130 aa (1). In the mCIITA-7 cDNA, the first ATG codon of the long open reading frame occurs at nucleotide 94, while a second potential start site occurs in the same reading frame at bp 166. This is exactly analogous to the human cDNA sequence, where a start codon at nucleotide 116 begins the long open reading frame, and a second in frame ATG occurs at nucleotide 188. Although Steimle and colleagues (1993) raised the possibility that the mature human protein might begin at the second ATG, this appeared unlikely given the apparent conservation between the two deduced protein sequences in this region. This hypothesis was proven when genomic CIITA was cloned (6). When the full-length mCIITA-7 insert was subcloned into a eucaryotic expression vector and transfected into MHC class II negative BW5147 cells, the stably transfected cells

were found to stain positive for MHC class II by FACScan analysis (unpublished data).

The minimal transcriptional activation domain in hCIITA is composed of 89 aa and is present between residues 37 and 125 (2). The mouse CIITA-7 clone is 89% conserved in this region, indicating that it could also encode a minimal activation domain. The amino-terminal 30-160 aa of the human protein contains a region rich in acidic amino acids and this region has an overall identity of 80% with the mouse CIITA-7 clone. In the human, aa 50-137 are 30% aspartate/glutamate and mCIITA-7 also has 30% aspartate/glutamate in this region. Human CIITA is rich in proline/serine/threonine (39%) at aa 162-328, corresponding to residues 162-276 (42% pro/ser/thr) in the mouse. mCIITA-7 contains a deletion of 56 aa corresponding to amino acids 272-327 of the hCIITA. The carboxy-terminal regions of hCIITA and mCIITA-7 are also highly conserved (86%, corresponding to aa 903-1130 of the human and aa 859-1078 of the mouse).

A BLAST (NCBI) sequence homology search was performed by API using mCIITA-7. There were no mouse DNA sequences that had conserved homology in the database (other than the partial codons mentioned above). Since our publication, several other DNA sequences for CIITA have been added, including the partial genomic sequences. Amino acid analysis and secondary structure predictions of mouse CIITA-7 were run using SEQSEE(PENSE) and PPSP at the Alberta Peptide Institute. It was found that mCIITA-7 contains 74 potential phosphorylation sites. In addition, the predicted structure of mCIITA-7 has a high

helical content and predicted protein motifs include a Bipartite Nuclear Targeting sequence (aa 144-161), an ATP/GTP binding site (365-376 aa), and numerous glycosylation sites. Of note, the conserved ATP/GTP binding site also occurs in human CIITA (aa 420-427). Data from this search indicate that mCIITA-7 is composed of two large domains separated by a long collagen-like helical domain. A BLAST network homology search matched residues 210-307 (proline-rich) with several collagen-like sequences and gave the largest number of matches, but no homologies (other than human CIITA) are displayed when the database is searched using the protein in its entirety. In addition, neither the amino-terminal acidic region (aa 1-320) nor the carboxyl-terminal region (aa 320-1082) were similar to any other known protein sequences (Table 4.1).

The proline-, serine-, and threonine- rich domains may be important to maintain an optimal conformation, although these regions are neither involved in transcriptional activation nor in providing MHC class II gene specificity. Zhou and Glimcher (1995) and Boss (1995) showed that hCIITA is an MHC class II gene-specific transcription activator which has gene specificity mediated by the carboxyl terminal residues (317-1130) and has transcription activation derived form the N-terminal acidic domain (aa 26-137). The conservation of the mouse amino acid sequence in the proline/serine/threonine region (12%) is lower than in other regions of the amino-terminal domain (80%) when compared to the human. The pro/ser/thr content is similar between mouse and human (42% vs 39%, respectively) indicating that the conformation of this region is likely conserved and important. The conformation of this putative collagen-like region may be

important to permit the N-terminal activation domain to contact the general transcription factors when the C-terminal specificity domain is bound and orientated by the proteins occupying the class II promoter (Figure 4.2).

The carboxyl-terminal region of CIITA may bind to more than one DNA-binding protein--proteins, which have already occupied the class II promoter, specifically binding the X1, X2, and Y boxes. Proteins that bind the W or other upstream elements may also influence promoter activity, suggesting that many interacting DNA-binding proteins stabilise a 3-dimensional complex (2). Indeed, when we compare CIITA of mouse and human in the carboxy-terminal region, mCIITA-7 reflects two regions of high homology (aa 317-779 and aa 846-1130) and a stretch of amino acids that show little or no homology to the human (aa 780-845). Perhaps these two regions correspond to two distinct sites for engaging proteins bound to the class II promoter.

The identification of the mouse CIITA cDNA and its similarity to the human contributed to the knowledge of the potential protein structure. The GTP/ATP binding site has since been shown to bind GTP, which is integral to the function of the protein (7).

At the same time I was characterising my cDNA clones, CIITA gene-disrupted mice were generated by Flavell's group (8). The gene disruption was made without the mouse CIITA sequence and using the human primers to construct the mouse targeting vector. This indicates that the conservation between the species is high and that the two genes serve very similar functions, namely to regulate class II expression. Both humans and mice deficient in CIITA

have severe defects in class II expression, indicating that the species similarities go beyond the sequences and directly influence immunity.

V. TABLE

Table 4.1: Sequence matches from the SEQSEE program for mouse CIITA clone 7. BNTis a putative nuclear targeting sequence. The GTP binding site has been shown to be important for nuclear targeting of CIITA. Leucine-rich motifs are associated with protein interactions.

Sequence Matches	Location (aa mCIITA)
• **BNT sequence**	• 144-161
• **ATP/GTP-binding site**	• 365-376
• **Glycosylation sites**	• 258-263 322-327 972-977 977-982
• **Potential Histone DNA recognition/binding site**	• 687-692
• **Leucine-rich repeat**	• 968-1017

VI. FIGURES

Figure 4.1: Alignment of mouse and human CIITA cDNA sequences. Mouse CIITA-7 (m) and human CIITA pDVP10-1 (h) are aligned and bold black lines indicate identical amino acid composition. Dashes indicate possible deletions based on misalignment.

```
 m1 MRCLVPGPSG SYLPELQDHS LCATMDLGSP EGSYLELLNS DADPLHLYHL YDQMDLAGEE
 h1 MRCLAPRPAG SYLSEPQGSS QCATMELGPL EGGYLELLNS DADPLCLYHF YDQMDLAGEE

m61 EIELSSEPDT DTINCDQFSK LLQDMELDEE TREAYANIAE LDQYVFQDTQ LEGLSKDLFI
h61 EIELYSEPDT DTINCDQFSR LLCDMEGDEE TREAYANIAE LDQYVFQDSQ LEGLSKDIFK

m121 EHIGAEEGFG ENIEIPVEAG QKPQKRRFPE EHAMDSKHRK LV-PTSRTSL NYLDLPTGHI
h121 -HIGPDEVIG ESMEMPAEVG QKSQKRPFPE ELPADLKHWK PAEPPTVVTG SLLVGPVSDC

m180 QIFTTLPQGL WQISGAGTGL SSVLIYHGEM PQVNQVLPSS SLSIPSLPES PDRPGSTSPF
h180 STLPCLPLPA LFNQEPASGQ MRLEKTDQIP MPF----SSS SLSCLNLPEG PIQFVPTIST

m240 TPSAADLPSM PEPALTSRVN ETEDTSPSPC QEGPES---- ---------- ----------
h236 LPHGLWQISE AGTGVSSIFI YHGEVPQASQ VPPPSGFTVH GLPTSPDRPG STSPFAPSAT

m276 ---------- ---------- ---------- --SIKLPKWP EAVERFQHSL QDKYKALPQS
h296 DLPSMPEPAL TSRANMTEHK TSPTQCPAAG EVSNKLPKWP EPVEQFYRSL QDTYGAEPAG

m304 PRGPLVAVEL VRARLERGSN KSQERELATP DWTERQLAHG GLAEVLQVVS DCRRPGETQV
h356 PDGILVEVDL VQARLERSSS KSLERELATP DWAERQLAQG GLAEVLLAAK EHRRPRETRV

m364 VAVLGKAGQG KSHWARTVSH TWACGQLLQY DFVFYVPCHC LDRPGDTYHL RDLLCPPSLQ
h416 IAVLGKAGQG KSYWAGAVSR AWACGRLPQY DFVFSVPCHC LNRPGDAYGL QDLLFSLGPQ

m424 PLAMDDEVLD YIVRQPDRVL LILDAFEELE AQDGLLHGPC GSLSPEPCSL RGLLAGIFQR
h476 PLVAADEVFS HILKRPDRVL LILDAFEELE AQDGFLHSTC GPAPAEPCSL RGLLAGLFQK

m484 KLLRGCTLLL TARPRGRLAQ SLSKADAIFE VPSFSTKQAK TYMRHYFENS GTAGNQDKAL
h536 KLLRGCTLLL TARPRGRLVQ SLSKADALFE LSGFSMEQAQ AYVMRYFESS GMTEHQDRAL

m544 GLLEGQPLLC SYSHSPVVCR AVCQLSKALL EQGTEAQLPC TLTGLYVSLL GPAAQNSPPG
h596 TLLRDRPLLL SHSHSPTLCR AVCQLSEALL ELGEDAKLPS TLTGLYVGLL GRAALDSPPG

m604 ALVELAKLAW ELGRRHQSTL QETRFSSVEV KTWAVTQGLM QQTLETTEAQ LAFSSFLLQC
h656 ALAELAKLAW ELGRRHQSTL QEDQFPSADV RTWAMAKGLV QHPPRAAESE LAFPSFLLQC

m664 FLGAVWLAQC NEIKDKELPQ YLALTPRKKR PYDNWLEGVP RFLAGLVFQP RAHCLGALVE
h716 FLGALWLALS GEIKDKELPQ YLALTPRKKR PYDNWLEGVP RFLAGLIFQP PARCLGALLG

m724 PAVAAVADRK QKVLTRYLKR LKLGTLRAGR LLELLHCAHE TQQPGIWEHV AHQLPGHGTR
h776 PSAAASVDRK QKVLARYLKR LQPGTLRARQ LLELLHCAHE AEEAGIWQHV VQELPGRLSF

m784 LSVDRKQKVL ARYLKRLQPG TLRARQLLEL LHCAHEAEEA GIWQHVVQEL PGRLSFLGTR
h836 LTPPDAHVLG KALEAAGQDF SLDLRSTGIC PSGLGSLVGL SCVTRFRAAL SDT-------

m844 LMALWESLQQ QGEAQLLQAA EEKFTIEPFK AKSPKDVEDL DRLVQTQRLR NPSEDAAKDL
h889 -VALWESLRQ HGETKLLQAA EEKFTIEPFK AKSLKDVEDL GKLVQTQRTR SSSEDTAGEL

m904 PAIRDLKKLE FALGPILGPQ AFPTLAKILP AFSSLQHLDL DSLSENKIGD KGVSKLSATF
h948 PAVRDLKKLE FALGPVSGPQ AFPKLVRILT AFSSLQHLDL DALSENKIGD EGVSQLSATF

m964 PQLKALETLN LSQNNITDVG ACKLAEALPA LAKSLLRLSL YNNCICDKGA KSLAQVLPDM
h1008 PQLKSLETLN LSQNNITDLG AYKLAEALPS LAASLLRLSL YNNCICDVGA ESLARVLPDM

m1024 VSLRVMDVQF NKFTAAGAQQ LASSLQKCPQ VETLAMWTPT IPFGVQEHLQ QLDARISLR
h1168 VSLRVMDVQY NKFTAAGAQQ LAASLRRCPH VETLAMWTPT IPFSVQEHLQ QQDSRISLR
```

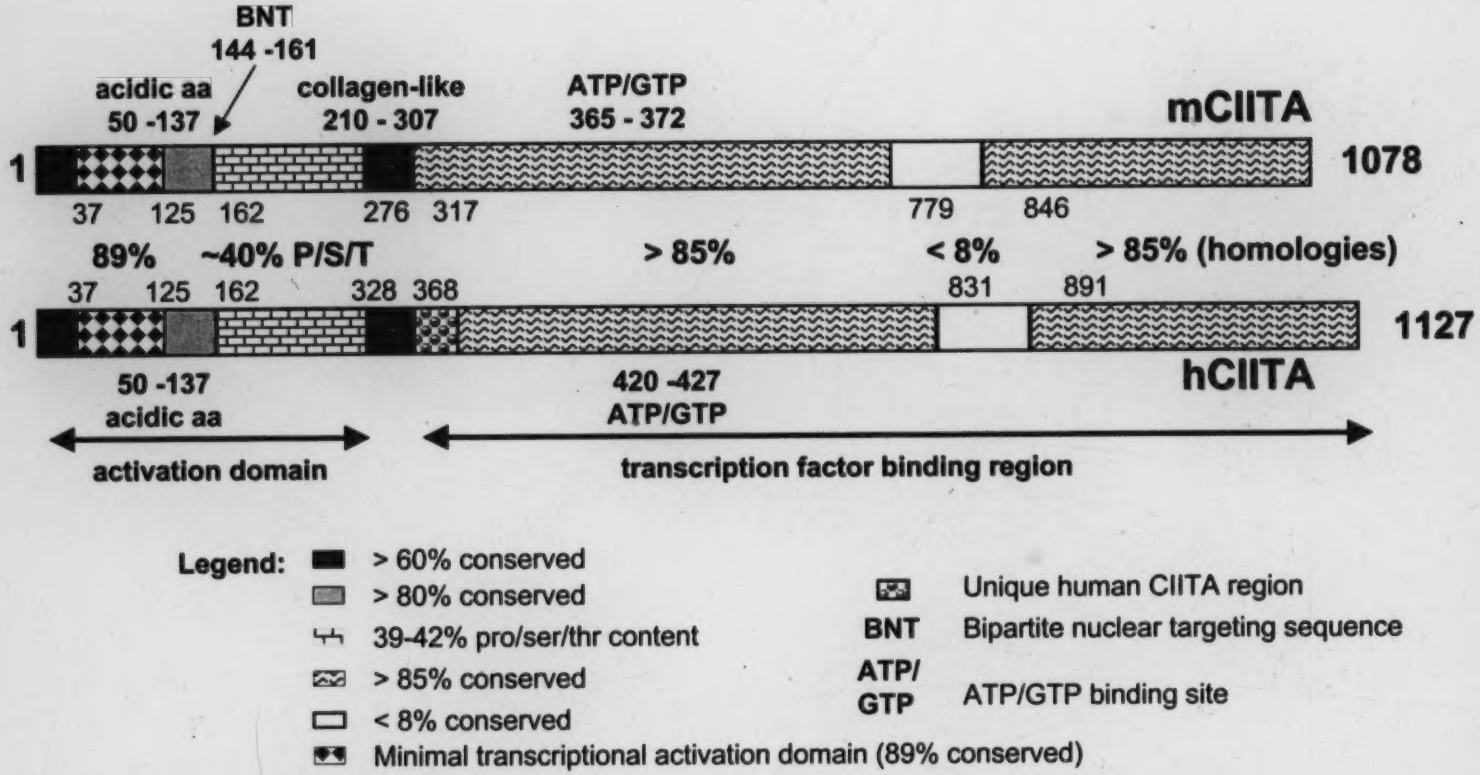

Figure 4.2 Alignment of mouse (m) and human (h) predicted amino acid sequences and conserved predicted domains for CIITA.

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CHAPTER 5

Class II Expression Is CIITA Dependent after Injury And Inflammation: Studies In The CIITA Knockout Mouse

Chapter 5

Class II Expression Is CIITA Dependent after Injury And Inflammation: Studies In The CIITA Knockout Mouse

(Tasha N. Sims, Joan Urmson, Lin Fu Xhu, and Philip F. Halloran. Contributions by other authors are noted)

I. PREFACE

CIITA is expressed only in cells that express class II such as "professional" antigen presenting cells (APC) or B-lymphocytes and in cells sensitive to IFN-γ stimulation such as the endothelium and peripheral epithelial cells. We showed that induction of CIITA mRNA expression accompanies the expression of class II after tissue injury (1). CIITA and class II mRNA are induced in kidney by acute tissue injury in wild-type, IFN-γ gene-disrupted mice, and IRF-1 gene-disrupted mice indicating that CIITA is inducible *in vivo* by non-IFN-γ stimuli. Mechanisms distinct from IRF-1 may participate in this induction since IRF-1KO mice also show reduced, but not absent, class II in response to injury. Therefore, I acquired mice deficient in the class II transactivator and investigated the regulation of MHC during inflammation, injury and graft rejection.

II. INTRODUCTION

H-2^b mice have a mutated Eα promoter and do not express I-Eα/β complexes on the cell surface. Class II KO mice (C57Bl/6, I-Aβ$^{-/-}$, H-2^b) have been generated previously (2;3) by introducing a deletion in the I-Aβ gene. These mice cannot express I-Aα/β complexes due to gene targeting and do not express I-E (since they are H-2^b). Therefore, they cannot express mature class II:peptide trimers on the cell surface. However they do have normal levels of I-Aα heavy chains sythesised. The contribution of these orphan molecules to the immune response is unknown since they will be produced at the same levels as in wild-type mice. It is likely that the heavy chains would be degraded in the ER and could thereby be processed and presented in the class I pathway. On the other hand, CIITAKO mice are an excellent tool to assess the role of both the class I and II gene family during an immune response *in vivo* because CIITA controls much of the class II antigen presentation machinery, even in non-professional APC (4). Thus CIITAKO mice are essentially class II heavy-chain negative.

Increased MHC expression in kidney is associated with autoimmunity, delayed graft function, tissue injury and graft rejection (5-9). We have shown that CIITA is increased at the steady state RNA level during tissue injury, allogeneic stimulation and inflammation (1;10). Thus I hypothesised that the lack of CIITA would inhibit MHC expression *in vivo* in response to various stimuli. I used CIITA gene-disrupted (CIITAKO) mice to investigate the roles for MHC after

inflammatory stimuli, allogeneic cell stimulation, tissue injury and allogeneic transplantation. I found that CIITA is absolutely required for class II upregulation in response to all systemic stimuli tested. In addition, I found no role for CIITA in class I regulation *in vivo*. In response to injury, CIITA is absolutely required for class II regulation. In our renal allograft model, CIITA in the graft is not required for kidney rejection.

III. MATERIALS AND METHODS

CIITAKO mice. CIITAKO mice were generated by R.A. Flavell (Yale University, New Haven CT) (11) and were a generous gift of C. H. Chang (University of Michigan, Ann Arbor, MI). Mice were maintained at the HSLAS at the University of Alberta.

Wild-type mice. CBA (H-2^k), 129/J and 129/J X B6 (F1) (H-2^b) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained at the HSLAS at the University of Alberta. 129/J, C57Bl/6 and 129/J X B6 (F1) mice were utilised as the controls for the CIITAKO mice.

P815 tumour allografts. (same as in chapter three). The murine mastocytoma tumour cell line P815 (American Type Culture Collection, Rockville, MD) was passaged in DBA/2 (H-2^d) mice from the Jackson Laboratory (Bar

Harbor, ME). After P815 cells were collected from the ascites fluid, 20 x 10^6 cells were injected i.p. into the mice. The tissues were harvested on day 7.

LPS injection. (Same as in chapter two) LPS of *Salmonella minnesota* (Sigma, St. Louis, MO) was dissolved in sterile saline at 250 μg/ml and heated at 56°C for 5 hours. Mice were injected i.p. with either saline (control) or LPS (25 μg) and were harvested either at various time points.

Acute tubular necrosis (ATN). (Same as in chapter three) Unilateral ischemic injury has been widely reported (12-14). Briefly, mice 8-12 weeks of age were anaesthetised with 2,2,2-tribromoethanol in tert-butyl alcohol (Avertin) by i.p. injection. The left renal pedicle was identified through a midline incision and occluded with a micro-bulldog clamp for 60 minutes. Before closure the kidney was inspected to ensure reperfusion, and the abdominal cavity was filled with warm saline. Each group had between 4 and 8 mice and the tissues were harvested at day 7. Control mice were normal mice.

Renal Transplants. Lin Fu Xhu performed the transplant surgeries. CIITAKO or 129/JxB6 F1 (H-2^b) mice were used as kidney donors into CBA (H-2^k) hosts. The mice were premedicated with Atropine i.m. and anaesthetised with pentobarbital i.p. The aorta above the renal artery was clamped and cannulated and then flushed with Ringer's solution. The kidney was excised from the donor and held at 4°C until transplanted. In the host, the right native kidney was

excised and the donor kidney and ureter was implanted into the host. Mice were maintained at the HSLAS at the University of Alberta and were given Buprenorphine for two days to alleviate pain and Ancef for three days as a prophylactic antibiotic. Transplanted right kidneys as well as the host left kidney and heart were harvested at day 7. Tissue samples were obtained for Periodic Acid Schiff (PAS) and routine histology, as well as indirect immunoperoxidase (IIP) staining for class I and II antigen expression.

Nothern blot. Tissues were homogenised in pairs with a polytron in 4 M guanidinium isothiocyanate and the RNA was pelleted through a 5.7 M $CsCl_2$ cushion (15). Northern blots were prepared by using 8 μg of total grouped (5-10 mice) RNA electrophoresed through a 1.5% agarose gel in the presence of 2.2 M formaldehyde. Samples were run overnight at 12-18V and a photo was taken the next day. The samples were transferred in 10X SSC to nitrocellulose and baked for 1.5 hours at 80°C. The filters were bathed in prehybridisation solution (16) containing 5X dextran sulfate and 50% formamide for 4 hours. Blots were then hybridised sequentially with ^{32}P-labelled cDNA probes (Klenow enzyme, Roche, Laval, PQ) for class I (HLA-A3) then class II (I-Aα). Each hybridisation was followed by exposure to Kodak X-Omat AR film at -70°C with an intensifying screen. Blots were stripped in base between hybridisations with new probe.

Antibodies. Monoclonal antibodies (mAb) were purified in our laboratory from supernatants of hybridoma cell lines. The lines were AF 6-120.1.2 (AF,

mouse IgG against mouse I-A^b), 20-8-4S (mouse IgG against mouse H-$2K^bD^b$), M1/42.3.9.8 (rat IgG_{2a} against all mouse H-2 haplotypes) and M5/114.15.20 (rat IgG_{2b} against mouse I-$A^{b,d,q}$ and I-$E^{d,k}$), obtained from American Type Culture Collection (Rockville, MD). Briefly, the hybridoma cell lines were maintained in tissue culture, and the supernatants containing AF 6-120.1.2 (anti I-A^b) and 20-8-4S (anti H-$2K^bD^b$) were purified by protein A chromatography. The supernatants containing M1/42.3.9.8 (anti H-2 antigens all haplotypes) and M5/114.15.20 (anti I-$A^{b,d,q}$ and I-$E^{d,k}$) were ammonium sulfate precipitated, and then the antibodies were purified with a DE52 anion exchanger column (Whatman, Hillsboro, OR) and by concentration with Amicon ultrafiltration. The protein concentration was adjusted to 1 mg/ml and maintained at -70°C. Peroxidase-conjugated goat IgG against rat IgG and peroxidase-conjugated goat IgG against mouse IgG were supplied by Organon Teknika (Scarborough, Ontario).

Radiolabelled antibody binding assay (RABA). (same as chapter two) Anti-H-$2K^bD^b$ mAb and anti-I-A^b mAb were radiolabelled with [^{125}I] iodide using the Iodogen method (Pierce Chemical Co., Rockford, IL). Tissues of individual mice were prepared as described previously (17). The tissue concentration was adjusted to 20 mg/ml. 5 mg of kidney tissue was aliquoted in triplicate and spun. The pellets were incubated on ice with ^{125}I-labelled mAb in 10% normal mouse serum (100,000 cpm per 100 μl) with agitation for 1 hour. After washing, the pellets were counted in a gamma counter and the non-specific binding of a negative tissue was subtracted. Statistical significance between experimental

groups was performed using ANOVA and MHC fold increases were determined from cpm counts using a standard curve. All RABA were done by Joan Urmson.

Staining of tissue sections. Flash-frozen cryostat sections were fixed in acetone, then incubated with normal goat serum. The slides were incubated with mouse mAb AF 6-120.1.2 (anti I-A^b) rat mAb against class I (M1) and class II (M5) or controls. The slides were then incubated with affinity purified peroxidase-conjugated goat anti rat IgG $F(ab')_2$ fragment. Antibody binding was visualised by the use of 3'3 diaminobenzidine tetrahydrochloride and hydrogen peroxide for the colour reaction and then counterstained with hematoxylin. Tissues were stained with the assistance of Joan Urmson.

IV. RESULTS

MHC is dependent on CIITA in the basal (unstimulated) state. We previously showed that class I levels are dependent on both IFN-γ and IRF-1 in the basal state. Class II expression is less dependent on these factors, but is also slightly reduced (not statistically significant) in the basal state in mice deficient in these factors (12;18). In the kidney, class I is expressed on the arterial endothelium and in the glomeruli while class II is expressed only on the interstitial cells (12). I examined MHC expression by immunohistochemistry in the kidney using rat MI and M5. Class II is not expressed in the kidney in the basal state in the CIITAKO mice, whereas the wild-type mice showed staining in the interstitial cells (Figure 5.1). The difference in class I expression between the two mouse strains in the basal state was apparent in class I staining of kidney sections (Figure 5.1). In the kidney, class I is usually detectable on the arterial endothelium and in the glomeruli in wild-type mice. However, in the CIITAKO mice, class I was also detectable on some tubules. Thus the increased level of class I in the kidneys of the CIITAKO mice is reflected in diffuse tubular class I expression compared to the wild-type mice. These data indicate that CIITA is completely required for basal expression of MHC class II and the lack of CIITA does not result in a loss of class I expression.

By radiolabelled antibody binding assay, basal expression of class I in CIITAKO mice was significantly higher compared to the wild-type mice (N=22) (Figure 5.2). Unexpectedly, the I-A^b mAb showed expression of class II in kidney homogenate in both wild type and CIITAKO mice (N=22). We investigated this reactivity further by staining tissue sections with the same mouse monoclonal used in the RABA. I found medulary staining in all mice including CIITAKO mice with this mouse monoclonal antibody (Figure 5.3). From these sections it was difficult to determine if there were strain differences in the non-specific staining. In the cortex, the staining is localised to the artery myocyte layer and adventitia (Figure 5.4). In the basal state, these structures are not known to express class II. Thus the I-A^b mouse monoclonal antibody has a cross reactivity and is not used for tissue sections. Unfortunately, the rat monoclonals cannot be used in the RABA due to lack of purity and the Serotec mouse I-Ab mA^b does not radiolabel (personal communication, Joan Urmson), leaving us with the AF antibody for the RABA. To determine if there was message for class II, I did northern blots for class II (discussed later) and found that there was no message for class II in the basal or induced states in CIITAKO mice in kidney or spleen. Together the data suggest that there is no class II in the basal state in the CIITAKO mice. I hypothesised that the increases in class I in CIITAKO mice were due to high IFN-γ levels. This question could be addressed by locally neutralising IFN-γ with an infusion of anti-IFN-γ antibody.

MHC class II levels are CIITA-dependent in response to systemic stimuli.

Bacterial LPS: Endotoxin injected into a mouse causes an increase in both MHC class I and II by day 3 in the kidney and these levels are maintained for up to seven days. The increases in class I and II are completely dependent on both IFN-γ and the transcription factor IRF-1 (12;18). I examined whether the increases in class I and II were dependent on CIITA in this model system. I injected WT and CIITAKO mice with heat-inactivated LPS and measured the levels of class I and II in kidney homogenate (Figure 5.5). Class I was increased approximately 8 fold by day 4 in WT and CIITAKO mice. However, in CIITAKO mice class II was absent throughout the experiment.

Allogeneic (P815) cells: We have shown that systemic stimulation by allogeneic cells massively increases both MHC class I and II in the kidney at day 7, and that the induction was completely dependent on IFN-γ and IRF-1 (12;19). I injected P815 cells into both WT and CIITAKO mice and harvested the kidneys at day 7 (Figure 5.6). WT mice showed strong induction in both class I (approximately 16-fold) and II (approximately 7-fold) levels as measured by radiolabelled antibody binding assay. In CIITAKO mice there was also a strong induction of class I, but of interest, there was no increase in class II. By immunohistochemistry, these increases in class I and II in the wild-type mice are mainly seen in the tubular epithelium (not shown). From northern blots (Figure 5.7) there is induced class I in the P815 group from wild type and CIITAKO mice

but no class II in either the basal state or the induced state in kidney or spleen in the CIITAKO mice. Thus the induction of class II in the kidney by allogeneic cells is completely dependent on CIITA.

MHC levels in response to tissue injury. In response to local renal ischemic injury, MHC levels are increased approximately two to three fold in the injured kidney. These increases are partly dependent on IFN-γ and IRF-1 and partly independent of these factors (1;12). We have previously shown that CIITA mRNA levels are markedly increased in response to injury. I questioned whether the injury response was completely dependent on CIITA or if there was a portion of the response that was independent of CIITA. I cross-clamped the left renal pedicle for 60 minutes in both WT and CIITAKO mice and harvested the organs at day 7 (Figure 5.8). The injured left kidneys of the WT mice showed a two-fold increase in both class I and II over the uninjured kidney, while the CIITAKO mice showed a two-fold increase in class I but not in class II.

Pathologist Dr. Marjan Afrousian assessed these kidneys (20;21). Together, we saw no difference in the degree of injury or infiltrate in the left injured kidneys of the WT and CIITAKO mice (Figure 5.9). A very mild infiltrate is present in both WT and CIITAKO mice. Both types of mice show dilated tubules with a loss of brush border, some caste formation, and some tubules have bare basement membranes in the injured kidney. There were also signs of epithelial cell flattening and sloughing in the left kidneys of both types of mice.

I stained the kidneys for class I and class II molecules. In the basal state, the wild-type mice showed weak arterial and interstitial staining for class I (Figure

5.10), similar to our earlier experiments (12). Similarly, the CIITAKO mice had weak arterial staining but also had diffuse tubular staining. For class II (Figure 5.10), wild-type mice showed interstitial staining and the CIITAKO mice showed no class II staining. The WT and CIITAKO mice had increased class I staining on the tubules, the endothelium, and on the interstitial cells in the injured kidney. Class II staining in the WT mice was on the tubules, arteries and in the interstitium of the injured kidney. There was no class II staining in the CIITAKO mice. Thus increases in MHC class I in response to tissue injury use non-CIITA mechanisms while increases in class II are completely dependent on CIITA.

MHC levels in response to transplantation. In response to transplant rejection across MHC differences, MHC levels are massively increased by day 5 (22). These increases are dependent on local IFN-γ production (23). I tested the hypothesis that the lack of CIITA would be protective to an allograft since there would be no mechanism of direct recognition of the graft by CD4+ T cells. We performed vascularised renal transplants across MHC differences using CBA mice (H-2^k) as the hosts and either 129xB6 F1 (control) mice or CIITAKO mice as right kidney donors. On day 7, renal grafts from CIITAKO mice behaved similarly to the WT allografts and had undergone rejection. From the radiolabelled antibody binding assay of renal homogenates for host MHC haplotypes, host class I and class II levels were induced systemically as measured by host MHC levels in the native kidney and by the host MHC type infiltrate in the transplanted kidney (Figure 5.11A). Donor MHC class I was also

increased in both the CIITAKO and control wild-type F1 grafts (Figure 5.11B, Table 5.1). MHC class II levels were induced in the F1 grafts (Figure 5.11B) and in the infiltrating host cells (Figure 5.11A) but not in the CIITAKO grafts (Figure 5.11B). Thus, CIITA is required for induction of class II even during a complex allograft rejection response and class I levels are not affected by the lack of CIITA.

V. DISCUSSION

I studied the requirement for CIITA in the induction of both class I and class II the basal state, in the response to inflammatory stimuli and during the injury response. I also investigated the role for CIITA in a vascularised allograft model. I found that CIITA was absolutely required for all class II induction regardless of the context of stimulation. Earlier studies in other strains of CIITAKO mice demonstrated that CIITA is not required for class I expression (11;24). Of interest, I found no role for CIITA in the induction of class I under any circumstance including complex responses such as injury and graft rejection; and, I found that the basal level of class I was higher in the CIITA deficient mice. Surprisingly, I have also shown the absence of CIITA has no protective effect during graft rejection.

In the basal state, class II message is not detectable in the kidney of CIITAKO mice. In the basal state, MHC is expressed on distinct cell types in the kidney. In wild-type mice, MHC class I is detectable on the arterial endothelium

and in the glomeruli while class II is detectable only on the interstitial cells (12). In the CIITAKO mice, class I is detectable not only on the arterial endothelium and in the glomeruli but also on the tubules in the basal state. The novel tubular staining likely accounts for the significantly higher class I levels I see in the basal state in the CIITAKO mice. The reasons for this increase in class I tubular staining in the CIITAKO mice are not clear.

Here I show that the class I response to systemic stimuli such as heat-inactivated LPS and allogeneic cells is completely independent of CIITA and the class II levels are completely dependent on CIITA. Systemic stimuli increases MHC levels in the kidney through IFN-γ and IRF-1 dependent mechanisms (12;18). Class I levels are completely dependent on these factors while class II is only partly dependent on IRF-1 but completely dependent on IFN-γ. These results are similar to results that another group has found with IFN-γ stimulation (24).

Our ischemic renal injury model induces class I and class II 2-3 fold in wild-type mice in the kidney (1;12). We showed that mice deficient in IFN-γ or the transcription factor IRF-1 had reduced class I and class II levels in kidney compared to wild-type mice after ischemic injury. However, there are components of the MHC response to injury that were independent of IFN-γ and IRF-1 (1;12). Class II is expressed in the absence of CIITA in a few cells, including some dendritic cells, and the injury response is a complicated response likely linked to healing in a tissue. Thus I questioned whether the MHC response to injury required CIITA. I wondered if class I was partly dependent on CIITA

during this complex injury response, since it is partly dependent on both IFN-γ or IRF-1. However, class I was completely CIITA-independent even in the injury response. I also demonstrated no increase in class II levels in response to injury in the CIITAKO mice. This is contrary to our expectations since the ischemic injury response is not an immune mediated response but rather is a non-specific local injury that may be mediated in part by the MAP kinase family (25). Here I show that a competent immune system is not required for the injury response since CIITAKO mice are severely immune compromised, lacking not only the class II antigen presentation but also CD4 T cells (11). Of interest, even though there was no class II expressed in the kidney of the injured mice, the kidneys were pathologically indistinguishable from the wild-type kidneys indicating that class II expression is affected by injury but is not a causative agent in the injury response. It is of particular interest that all class II responses are controlled by CIITA, even during non-immunologic stimulation. This indicates that the promoters of CIITA may be sensitive to environmental stimuli such as changes in the extra cellular matrix and the stress response.

During graft rejection, class I and II molecules are massively induced both in the rejecting organ and in the host organs. Class II positive interstitial cells are reduced in the host organs while the transplanted organ becomes massively infiltrated with mononuclear cells that are class I and II positive. Two pathways of antigen recognition are hypothesised to be important for graft recognition by the host. Direct recognition of foreign MHC class II by CD4 T cells involves host T cells responding to donor MHC on donor antigen presenting cells. On the other

hand, indirect recognition of foreign class II by host T cells involves the host APC presenting peptides derived from donor MHC in the groove of host MHC. The APC is central in the immune response against a graft since CD8 T cells require help from CD4 T cells in order to become effector cells (26-28). Cardiac grafts with "no" MHC were rejected by WT mice indicating that the indirect pathway was sufficient for graft rejection (29;30). But data from skin grafts in class II KO mice show that the indirect pathway was sufficient to mediate graft rejection. Class II KO mice express I-Aα and the entire class II presenting machinery, casting doubt as to the validity of the experiments in class II KO mice with respect to determining if the indirect or the direct pathway are required for graft rejection. Our data in a vascularised allogeneic transplant model indicate that the direct recognition of class II on the graft is not required for graft loss. Thus, the indirect pathway within the graft is sufficient for graft rejection.

Many studies *in vitro* have shown that CIITA regulates MHC class I and that CIITA is required for the vast majority of class II expression. Here I show that under no circumstances that I studied was class I expression lost in the absence of CIITA. These data have implications for the regulation of class II during a normal immune response in non-lymphoid tissues. I demonstrated that CIITA is an integral molecule for the induction of class II in the basal and induced states including during injury and the regulation of class II during organ transplantation.

VI. TABLE

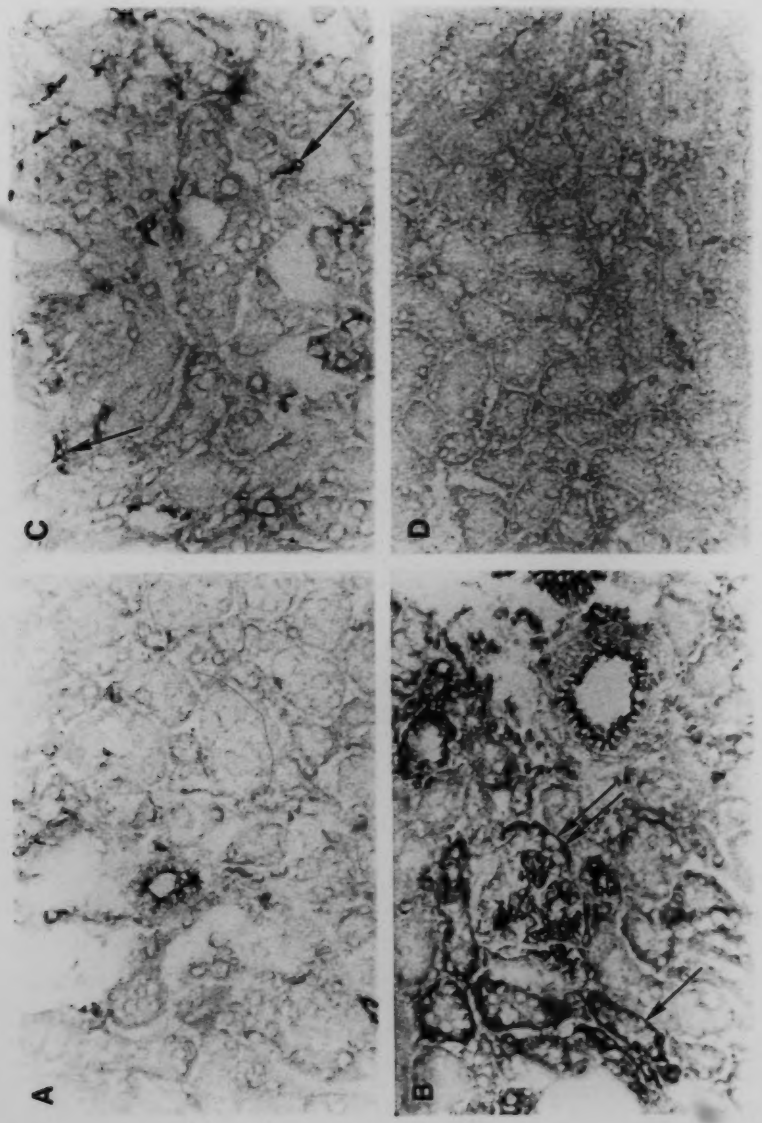
Table 5.1: Approximate fold increase of MHC molecule levels above baseline* .
Data taken from experiments show, numbers based on a standard curve.

		Class I		Class II	
	mice:	WT	CIITAKO	WT	CIITAKO
*Treatment^*					
LPS		7	7	2	0.5
P815		15	12	6	1
ATN		3	3	3	1
Tx (donor)		9	12	9	1

*compared to sham injected mice of same strain, as determined by RABA
^ LPS, lipopolysaccharide; P815, allogeneic cells; ATN, ischemic renal injury; Tx, transplant

VII. FIGURES

Figure 5.1: Photomicrographs of basal MHC levels in kidney. Frozen sections were stained with, panels A and B, M1 (anti-class I) or, panels C and D, M5 (anti class II) monoclonal antibodies. For class I, F1 wild type mice (A) show low levels of class I staining, mainly localised to the endothelium (arrow). CIITAKO mice (B) show diffuse tubular staining (arrow) and some glomeruli also stained for class I (double arrow). For class II, F1 wild type mice (C) show interstitial staining (arrow) while the CIITAKO mice show no class II staining (D). All photos taken at same magnification.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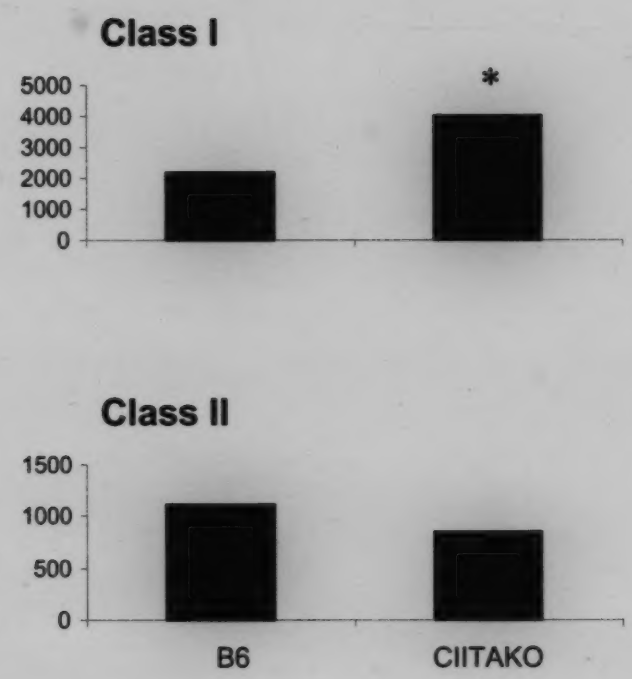

Figure 5.2: Basal MHC levels in the kidneys of CIITAKO and wild-type mice. Radiolabelled anti-class I or -class II antibodies were bound to normal renal homogenates and cpm measured. *Two-tailed T test $p \leq 0.01$.

Figure 5.3: Photomicrographs of non-specific medulary I-A^b staining in kidney from various mouse strains. Frozen sections were stained with mouse anti-I-A^b monoclonal antibody to determine staining patterns. Panel A, normal CBA; panel B, normal 129/J; panel C, CIITAKO; panel D, BALB/c. All panels are the same magnification (original 100x).

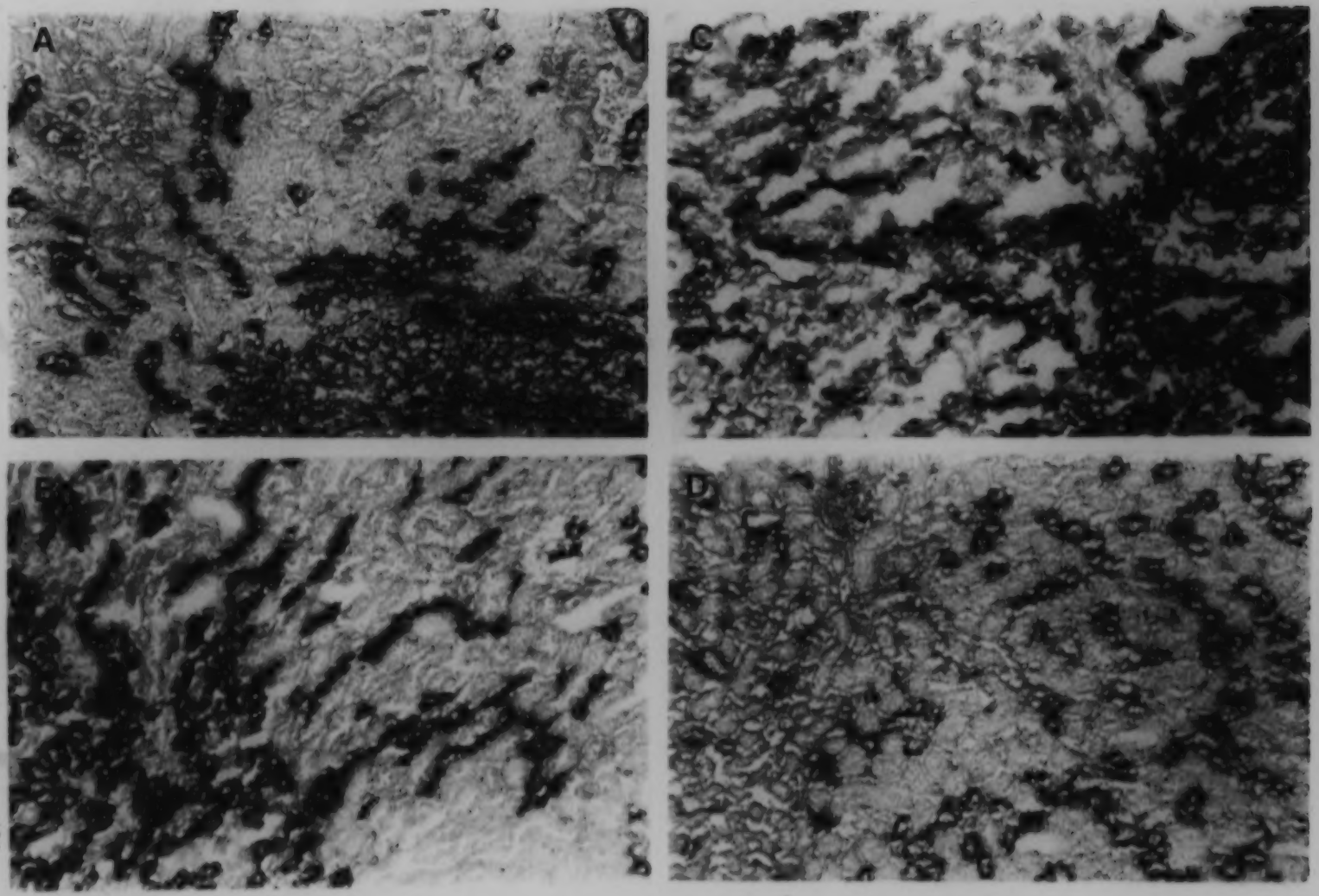
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Figure 5.4: Photomicrographs of non-specific I-A^b staining in kidney artery from various mouse strains. Frozen sections were stained with mouse anti-I-A^b monoclonal antibody to determine staining patterns. Arrow shows non-specific staining in the artery wall. Panel A, normal CBA; panel B, CIITAKO; panels C and D, normal 129/J BALB/c. All panels are the same magnification (original 160x).

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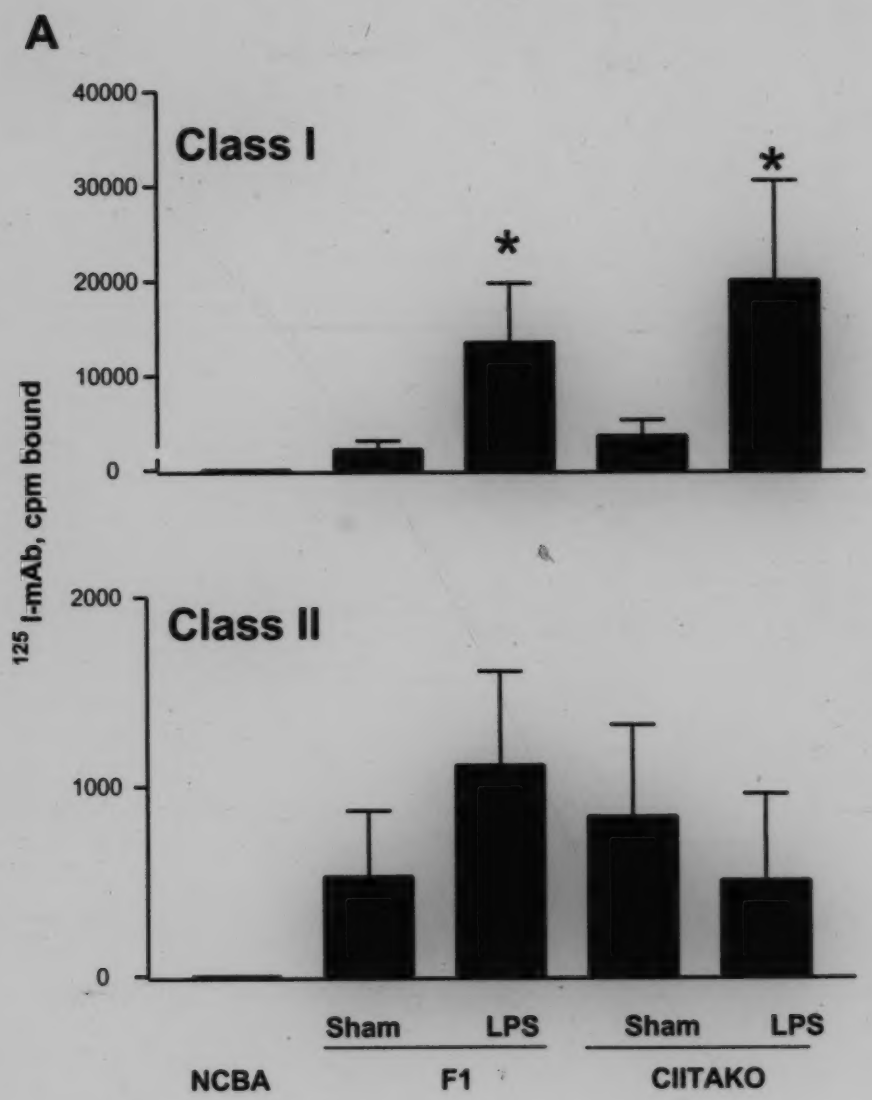


Figure 5.5: MHC levels in response to systemic stimuli. (A) CIITAKO or 129xB6 (F1) mice were injected i.p. with either saline or 25mg LPS and their kidneys were harvested at day 4. MHC levels were monitored by RABA. *ANOVA $p \leq 0.01$ compared to sham-injected mice in each group.

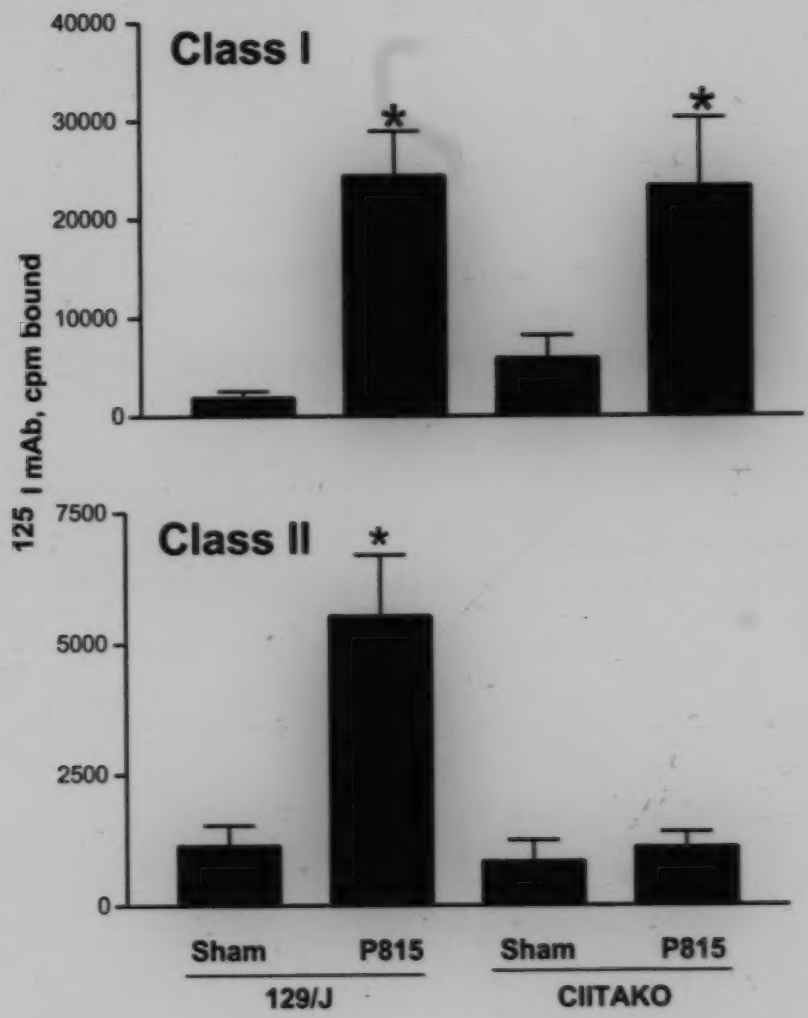


Figure 5.6: MHC levels in response to systemic (P815) stimuli. CIITAKO or 129/J mice were injected i.p. with either saline or P815 tumour cells and their tissues harvested on day 7. MHC levels were monitored by RABA. *ANOVA $p \leq 0.01$ compared to sham-injected mice in each group.

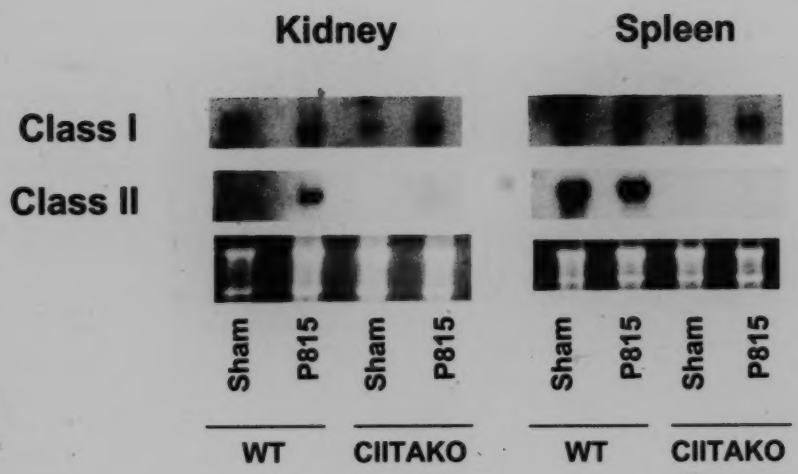


Figure 5.7: MHC mRNA levels in response to allogeneic (P815) stimuli. CIITAKO or WT mice were injected i.p. with either saline or P815 tumour cells and their tissues harvested on day 7. MHC mRNA levels were monitored by northern blot. Kidney, WT is 129xBl6 F1. Spleen WT is C57Bl/6.

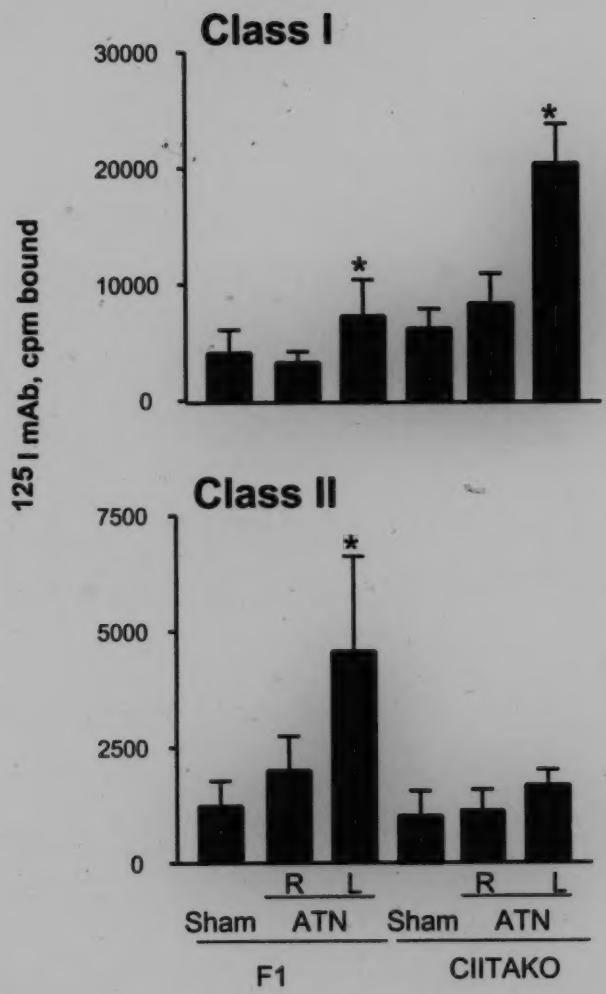
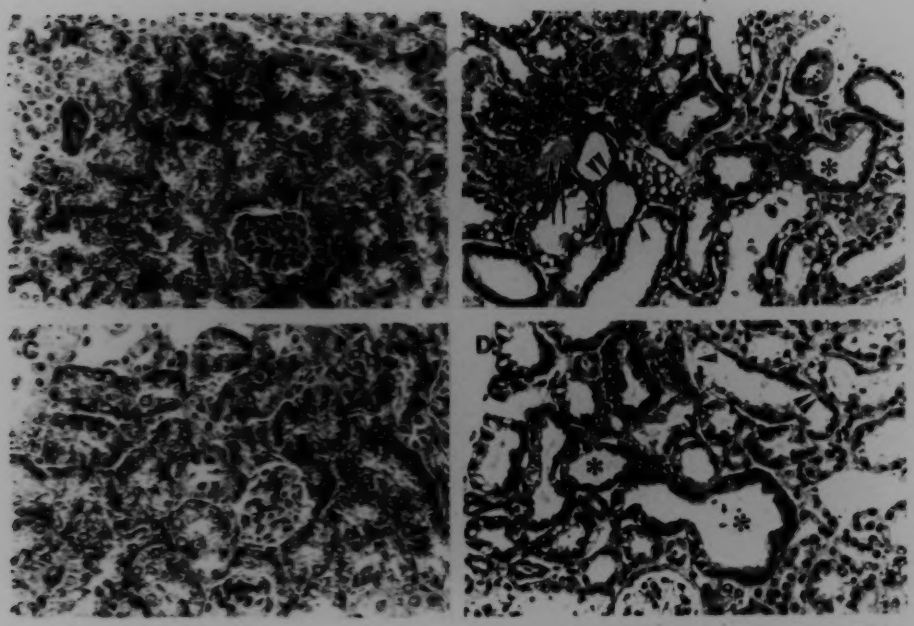


Figure 5.8: MHC induction in response to renal ischemic injury. CIITAKO or 129/JxB6 (F1) mice were injured by cross-clamping the left renal pedicle for 60 min. Both the injured left kidney and the contralateral right kidneys were harvested at day 7. MHC levels were monitored by RABA in both normal mice and injured mice. *ANOVA $p \leq 0.01$ compared to the right kidney in each group.

Figure 5.9: Histopathology of kidney after injury. CIITAKO or 129/JxB6 (F1) mice were injured by cross-clamping the left renal pedicle for 60 min. Both the contralateral right kidney (A, C) and the (injured left kidney B, D) were harvested at day 7. **Top set: hematoxylin-eosin (HE) staining**. The injured kidneys (B, D) of both F1 (B) and CIITAKO (D) mice show dilated tubules (asterix), caste formation (double arrows), flattened epithelial cells (double arrowhead) and epithelial cell sloughing (single arrowhead). **Bottom set: Periodic-Acid-Schiff (PAS) staining**. In the right kidney (A, C), brush border (arrow) is evident on the proximal tubules in both F1 (A) and CIITAKO (C) mice. In the left kidney (B, D) dilated tubules (asterix) and tubular congestion (arrow) are evident in both types of mice.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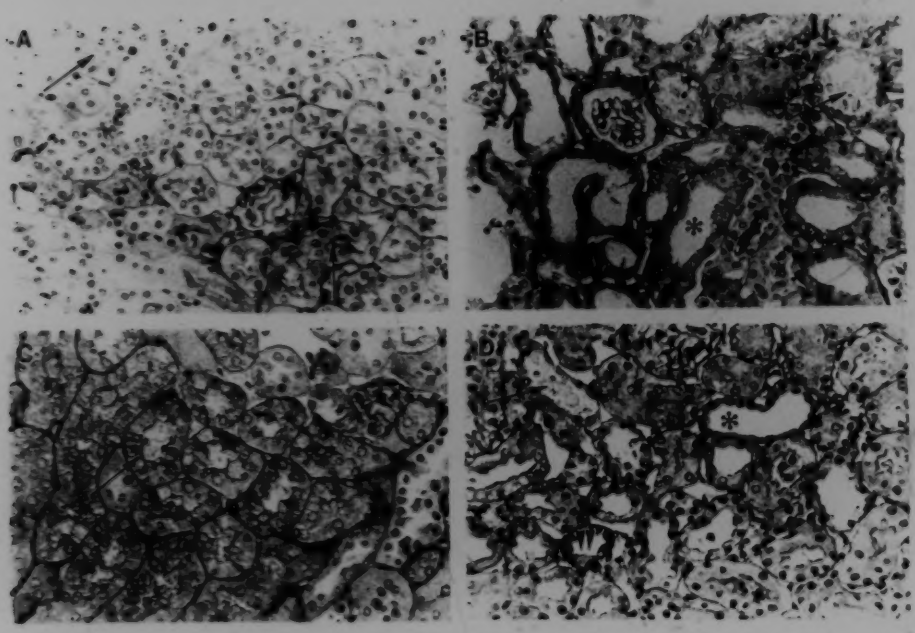
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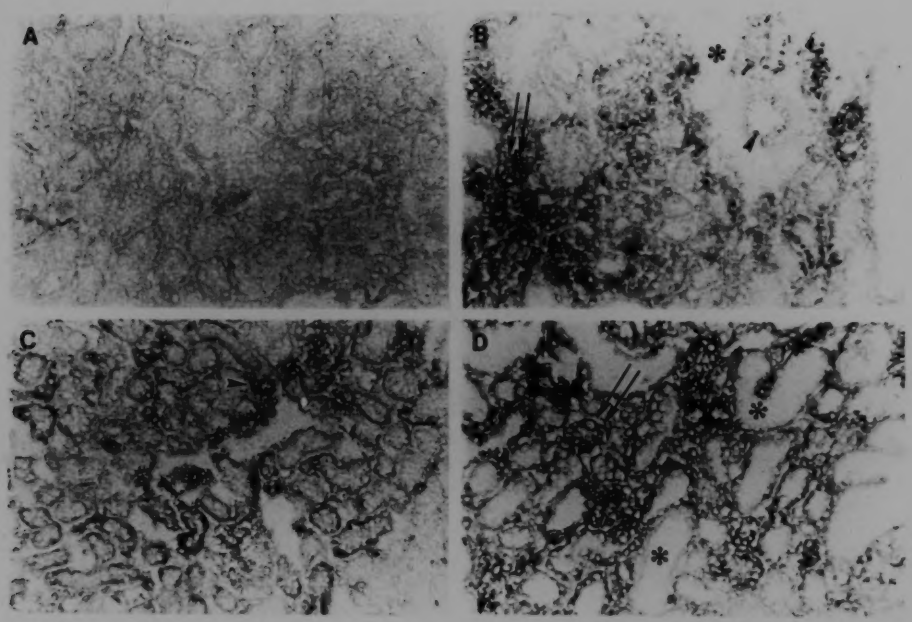
Figure 5.10: Photomicrographs of MHC class I and II levels in kidney after ischemic renal injury. CIITAKO or 129/JxB6 (F1) mice were injured by cross-clamping the left renal pedicle for 60 min. Both the contralateral right kidney (A, C) and the (injured left kidney B, D) were harvested at day 7. **Top set: Class I (M1) staining**. The injured kidneys (B, D) of both F1 (B) and CIITAKO (D) mice show dilated tubules (asterix), and strong tubular staining (double arrows). **Bottom set: Class II (M5) staining**. In the wild type right kidney (A) and injured left (B) kidney interstitial staining (arrow) is shown. (B) Tubular staining is also evident. The endothelium is not stained (arrowhead). CIITAKO (C, D) mice show dilated tubules (star) but no staining.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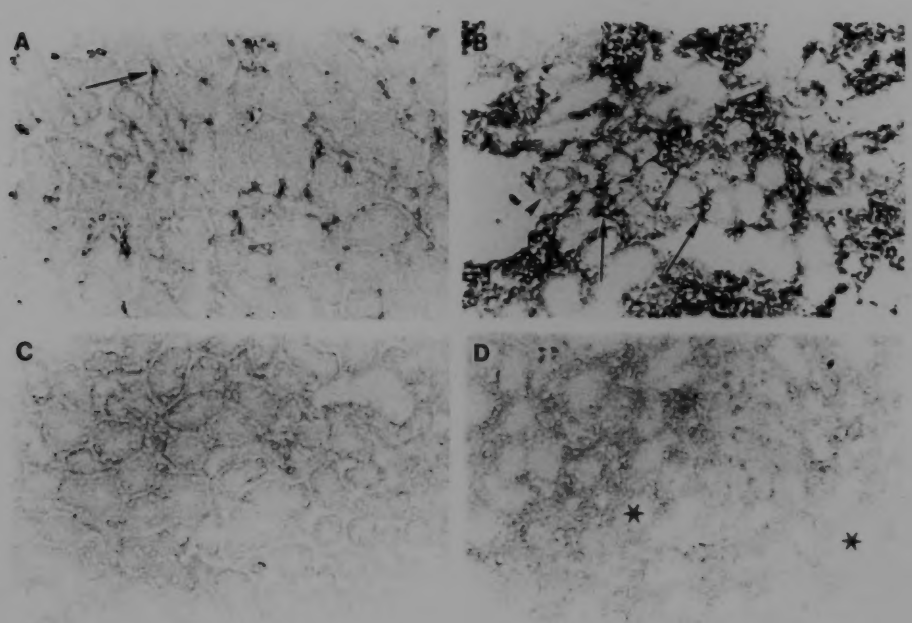
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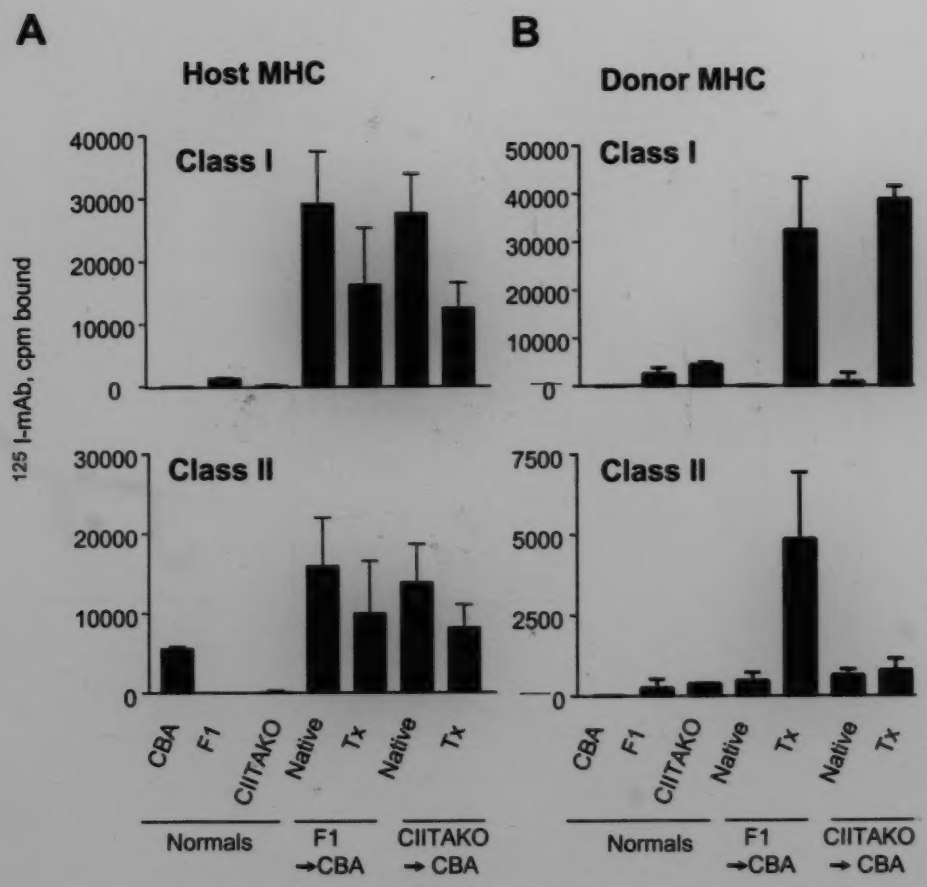

Figure 5.11: MHC expression in transplanted kidneys and hosts. Ten CBA mice were given either a CIITAKO or a 129 x B6 F1 (F1) kidney as an allograft. At day 7, the transplanted (Tx) kidneys and host (native) kidneys were harvested. MHC expression was monitored by RABA. **(A)** Host MHC ($H\text{-}2^k$) levels were monitored in the rejecting kidneys (infiltrate), host (native) and in normal kidneys **(B)** Donor MHC ($H\text{-}2^b$) levels were monitored in the rejecting kidneys (induction), host (native, infiltrate) and in normal kidneys.

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CHAPTER 6

The CIITA Promoters Are Differentially Regulated *In vivo* in the Basal State and After Inflammation and Injury.

Chapter 6

The CIITA Promoters Are Differentially Regulated *In vivo* in the Basal State and After Inflammation and Injury.

(A version of this chapter has been submitted for publication. Sims T.N. Takei, Y. Urmson, J., and Halloran, P.F. Conributions by other authors are noted).

I. PREFACE

The master regulator of constitutive and IFN-γ-inducible class II gene expression is the class II tranactivator (CIITA) (1-3). Although I was the first to clone the cDNA of mouse CIITA, the group lead by Bernard Mach in Basel described the promoters of CIITA in 1997. The regulation of CIITA is complex with four promoters described for the human and three for the mouse gene. The intricacies of the promoter system are elegant, with the various roles of class II accountable for by the combinations of promoter usage of CIITA. When the promoters were described, I went on to describe the relationship between CIITA control and the patterns of class II expression we have seen in the kidney *in vivo*.

II. INTRODUCTION

The complexity of the patterns of expression of class II makes the control of CIITA expression a critical point in the immune system, relevant to immune system development, maintenance, and responses. Thus considerable interest has focussed on the CIITA promoters (4). Cloning of the CIITA 5-prime (5') regulatory region revealed four non-homologous promoters in the human and three in the mouse (4-6). Promoter I (PI) and promoter III (PIII) are mainly detectable in dendritic-cell and B-cell lines, respectively, and are IFN-γ-independent *in vitro* (4). Promoter IV (PIV) has multiple potential transcription factor binding sites including γ-activated sites (GAS) and interferon stimulated response elements (ISRE), and is inducible by IFN-γ *in vitro* (4;7;8). The type II promoter is detectable only in the human CIITA gene (4) and its role is unknown.

The role of the CIITA promoters has been studied only in cell lines, leaving questions open about the *in vivo* role of these promoters in the whole animal. In the present experiments, I investigated the relative steady-state levels of each promoter-associated 5' untranslated mRNA and the level of total CIITA steady-state mRNA from the translated region in mouse tissues. By RT-PCR, CIITA mRNA levels of either the unique first exon (indicative of individual promoter usage) or downstream common exons (indicative of total full-length CIITA transcripts) were amplified. All the transcripts share a common open reading frame (4). To allow for semi-quantitative analysis of promoter usage, I cloned each promoter-associated PCR product and employed standard curves to

compare relative levels of promoter mRNA expression. I found that the basal and induced behaviours of the mRNA levels from each CIITA promoter confirmed the *in vitro* observations on the roles of the promoters but also suggest additional levels of control *in vivo*.

III. MATERIALS AND METHODS

GKO mice. (the same as chapter two). Heterozygous GKO mice were generously provided by Dr. T. Stewart (Genentech Inc., South San Francisco, CA). The GKO mice were created by disrupting the IFN-γ gene (9).

IRF-1 gene-disrupted (IRF-1 KO) mice (Same as chapter three). The homozygous IRF-1 KO mice, generously supplied by Dr. T. W. Mak (Ontario Cancer Institute, Toronto, Ontario), were generated by disrupting the IRF-1 gene (10).

Wild-type mice. BALB/c (BALB/cCr/AltBM) mice were obtained from the Health Science Laboratory Animal Services (HSLAS) at the University of Alberta. 129/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained at the HSLAS at the University of Alberta. BALB/c and 129/J mice were utilised as the controls for the GKO and the IRF-1 KO mice, respectively.

Recombinant IFN-γ treatment. The rIFN-γ was generously provided by Dr. T. Stewart (Genentech Inc., South San Francisco, CA). Mice received 100,000 IU of rIFN-γ in a single intraperitoneal (i.p.) injection and were killed either at 4 hours or at 2, 4, 12, 24, 48, 72, or 96 hours for the CIITA promoter activation time course.

P815 tumour allografts (The same as chapter three). We monitored the kidney during rejection of allogeneic ascites tumour cells (P815) in the peritoneal cavity to monitor systemic IFN-γ production during the allo-response. Allogeneic stimulation across minor H antigen or MHC differences induces massive IFN-γ release by day 7. The murine mastocytoma tumour cell line P815 (ATCC, American Type Culture Collection, Rockville, MD) was passaged in DBA/2 (H-2^d) mice from the Jackson Laboratory (Bar Harbor, ME). After P815 cells were collected from the ascites fluid, 20 x 10^6 cells were injected i.p. into mice. The tissues were harvested on day 7.

LPS (endotoxin) injection. LPS of *Salmonella minnesota* (Sigma, St. Louis, MO) was dissolved in sterile saline at 250 μg/ml and heated at 56°C for 5 hours. Mice were injected i.p. with either saline (control) or LPS (25 μg) and were harvested at 1, 3, 6, 12, 24, 48, 72, or 96 hours.

Acute tubular necrosis (ATN). Mice 8-12 weeks of age were anaesthetised with 2,2,2-tribromoethanol in tert-butyl alcohol (Avertin) by i.p.

injection. The left renal pedicle was identified through a midline incision and occluded with a micro-bulldog clamp for 60 minutes. Before closure the kidney was inspected to ensure reperfusion, and the abdominal cavity was filled with warm saline. Each group had between 5 and 10 mice and the tissues were harvested at day 7. Control mice were normal CIITAKO or wild type mice.

Antibodies. Monoclonal antibodies (mAb) were purified by Joan Urmson in our laboratory from supernatants of hybridoma cell lines. The lines were 25-9-17S (mouse IgG_{2a} against mouse I-A^d) and M5/114.15.20 (rat IgG_{2b} against mouse I-$A^{b,d,q}$ and I-$E^{d,k}$), obtained from American Type Culture Collection (Rockville, MD). Briefly, the hybridoma cell lines were maintained in tissue culture, and the supernatants containing AF 6-120.1.2 were purified by protein A chromatography. The supernatants containing and M5/114.15.20 were ammonium sulfate precipitated, and then the antibodies were purified with a DE52 anion exchanger column (Whatman, Hillsboro, OR) and by concentration with Amicon ultrafiltration. The protein concentration was adjusted to 1 mg/ml and maintained at -70°C. Peroxidase-conjugated goat IgG against rat IgG and peroxidase-conjugated goat IgG against mouse IgG were supplied by Organon Teknika (Scarborough, Ontario).

Radiolabelled antibody binding assay (RABA) (The same as chapter two). Anti-I-A^d mAb were radiolabelled with [^{125}I] iodide using the Iodogen method (Pierce Chemical Co., Rockford, IL) (11). Tissues of individual mice were

prepared as described previously (12). The tissue concentration was adjusted to 20 mg/ml. 5 mg of kidney tissue was aliquoted in triplicate and spun. The pellets were incubated on ice with ^{125}I-labelled mAb in 10% normal mouse serum (100,000 cpm per 100 μl) with agitation for 1 hour. After washing, the pellets were counted in a gamma counter and the non-specific binding of a negative tissue was subtracted. Statistical significance between experimental groups was performed using ANOVA. MHC fold increases were determined from cpm counts using a standard curve.

Staining of tissue sections. Flash-frozen cryostat sections were fixed in acetone, then incubated with normal goat serum. The slides were incubated with rat mAb against class II (M5) or controls. The slides were then incubated with affinity purified peroxidase-conjugated goat anti rat IgG F(ab')$_2$ fragment. Antibody binding was visualised by the use of 3'3 diaminobenzidine tetrahydrochloride and hydrogen peroxide for the colour reaction and then counterstained with hematoxylin. Tissue sections were stained by Joan Urmson.

RNA and RT-PCR. Total RNA was isolated and pooled from two mice by 4 M guanidinium isothiocyanate extraction followed by centrifugation in 5.7 M cesium chloride as described previously (12). Equal amounts of RNA from 5-10 mice were pooled and total RNA (10 μg) was electrophoresed in a 1.5% agarose gel in 2.2 M formaldehyde and was detected when stained with ethidium bromide. The remaining RNA was set aside for RT-PCR. RNA was reverse transcribed into

cDNA using Superscript reverse transcriptase (BRL, Burlington, Ontario). The cDNA was amplified in a Perkin Elmer Cetus thermal cycler using Taq DNA polymerase and each 5' primer specific for the CIITA mRNA first exon (as follows: CIITA mRNA second exon (common) antisense, TCG CAG TTG CCA CGG AGC TGG; PI sense, TGA CTT TCT TGA GCT GGG TCT; PIII sense, TAC TGC ATC ACT CTG CTC TCT; PIV sense, CCT AGG AGC CAC GGA GCT GG). Primers were designed by Y. Takei and primers for total CIITA were based on those previously shown to amplify human and mouse CIITA mRNA (1;13). The reverse transcription reaction was incubated at 42°C for 45 min. The thermocycler program consisted of an initial denaturation for 2 minutes at 94°C followed by 30 cycles of: denaturation for 30 seconds at 94°C, primer annealing for 30 seconds at 55°C, and extension for 1 minute at 72°C. The CIITA translated region primers amplified a 434 bp fragment as detected on Southern blot with a human CIITA cDNA probe, a gift from Dr. R. Flavell (Yale University, New Haven, CT), provided with the permission of Dr. M. Peterlin (Department of Medicine, UCSF, San Francisco, CA). The CIITA promoter primers amplified a single band of approximately 250 bp detectable on a 1.5% agarose gel stained with ethidium bromide and also upon hybridisation with the specific mouse CIITA mRNA first exon internal 5'-UTR probes *for specificity* [as follows: PI, CTG GTG CCC CTC TCT ACC GTG GGA GCC T; PIII, GTA AGA CCC AGA AGG GCC AGG AAC CAG G; PIV, CCG TGC TTC TGA GTG CTG CCT GCA TGC A (one figure shown, done by Y. Takei)]. *For quantitation* a common internal exon 2 AS probe was used (as follows: GAT CTC CTC CTC CCC AGC CAG GTC CAT G).

HPRT was amplified using specific primers: sense (5' GTT GGA TAC AGG CCA GAC TTT GTT G), antisense (5' GAG GGT AGG CTG GCC TAT GGC T) and an internal specific probe (5' GCA GGT GTT GAG TCC TGT GGC CAT CTG CCC TAG). Following hybridisation the blots were exposed to Kodak X-Omat AR film at -70°C with an intensifying screen. The amount of hybridisation was quantitated using a Phosphor Imager Type BAS-III S (Fuji photo film Co. Ltd, Japan). Phosphor Imaging units depend strength of signal, which is dependent on variables including: analysis area (mm^2) drawn for each sample box, length of blot exposure, efficacy of probe labelling etc. The analysis area, blot exposure and probe labelling were identical within each experiment, but differ among experiments. Thus we use arbitrary units for Imaging data. The RNA loading was monitored by probing for HPRT and was expressed as arbitrary Phosphor Image counts. Within promoter usage experiments all RT-PCR reactions, gel and hybridisation conditions, and exposure times were identical.

Evaluation of relative mRNA levels for the three transcripts. P815-induced spleen mRNA was RT-PCR amplified using the promoter primers described above. PCR products were cloned by TOPO TA cloning (Invitrogen, Carlsbad, CA) and the purified cloned DNA was quantitated. Molar amounts of each clone were used to generate a standard curve to assess the relative efficiency of each sense primer. Dilutions ranging from 1:100 to 1:2 were made.

IV. RESULTS

Relative efficiencies of the PI, PIII, and PIV mRNA amplification systems. I amplified and cloned the promoter-associated 5' untranslated regions of the CIITA mRNAs by RT-PCR (Figure 6.1A). I isolated each positive clone, quantitated the plasmid concentration and then used the PCR products as templates to assess the relative efficiency of each sense primer. I then developed standard dilution curves from Phosphor Imaging data and determined the relative efficiencies of the primers. The relative amplification efficiencies of PI, PIII and PIV were not discernibly different over multiple experiments (Figure 6.1B).

Expression of CIITA promoters in mouse tissues in the basal (unstimulated) state. We have shown that mouse CIITA mRNA expression *in vivo* correlates with the distribution and regulation of class II in non-lymphoid organs (14). I compared the amplified levels of promoter and total CIITA mRNA in tissues from mice in the basal state (Figure 6.2). PI usage is associated with class II expression in dendritic cells (4). The spleen, kidney, liver, heart, and brain expressed PI mRNA in the basal state. PIII, associated with expression in B cells, was strongly expressed in the spleen and poorly expressed in all non-lymphoid tissue, consistent with their low B cell content. PIV, the IFN-γ-inducible promoter, was markedly expressed in kidney, spleen and skin. Total CIITA correlated closely with the pattern of PIV usage. Looking at the kidney by semi-quantitative analysis, approximately 8-fold more PI and 5-fold more PIV than PIII

are expressed in the basal state. Thus in unstimulated tissues, promoter usage varies and total CIITA is differentially expressed.

Of particular interest, the IFN-γ inducible promoter PIV is utilised in the basal state. As shown in Table 1, basal mRNA levels for PI and PIV were strong in mice lacking IFN-γ or IRF-1. Thus the basal mRNA levels of PIV reflect induction by non-IFN-γ dependent mechanisms.

Class II expression in renal interstitial cells in the basal state is independent of IFN-γ (12). These interstitial cells are related to dendritic cells but lack the stimulatory function of mature dendritic cells (15). Thus PI expression in the basal state in kidney was expected and is compatible with the pattern of class II expression in interstitial cells. I investigated the role of IFN-γ in CIITA promoter expression in WT and GKO mice in the basal state (Tables 1 and 2). PI and PIV expression is not dependent on IFN-γ. Surprisingly, PIII mRNA levels were significantly higher ($p \leq 0.0001$) in the kidneys of GKO mice indicating either that IFN-γ normally suppresses PIII usage, and/or that IFN-γ mediates cellular population changes that alter PIII mRNA levels.

Relative effect of rIFN-γ administration on CIITA promoters in kidney. IFN-γ induces the expression of CIITA mRNA and class II mRNA and products *in vivo* (12;14) but IFN-γ has been shown to activate only PIV *in vitro* (4). To test whether IFN-γ regulates only PIV *in vivo*, we administered rIFN-γ and harvested the kidneys at times from 2 hours to 96 hours (Figure 6.3). rIFN-γ induced the

expression of class II product (as measured by radiolabelled antibody binding assay), peaking at day 2 (data not shown). The increase in total CIITA mRNA levels correlated with the increase in PIV usage. Both total CIITA mRNA (approximately 10-fold increase) and PIV mRNA (approximately 20-fold increase) levels were induced by rIFN-γ, peaking at 4 hours. PIII was not activated above baseline at any time. Unexpectedly, PI mRNA increased approximately 5-fold between 12 and 96 hours after rIFN-γ injection. The mechanism behind the PI increase is unclear.

The majority of class II protein expression induced in kidney by IFN-γ is located on the tubular epithelium (12). I studied whether IFN-γ affected the interstitial cell population. We stained tissue sections for class II (I-A and I-E) protein with M5 monoclonal antibody and counted M5-positive interstitial cells (Figure 6.4). The interstitial cell population contains the only class II positive cells in the basal state. The staining is lost by day 1 of stimulation with rIFN-γ, and the staining returned to unstimulated levels by 96 hours. These kinetics contrast with the expression on renal tubules, which are class II negative by immunohistochemistry in the basal state, stain intensely by day 1, and remain class II positive until approximately day 7 (12;14). Interstitial cells express class II independent of IFN-γ and this expression may depend on both PI and PIV since both are used in the basal state (Figure 6.2 and Table 6.2).

The relative role of IRF-1 in the basal state and rIFN-γ-induced expression of the CIITA promoters in kidney. IRF-1 deficiency does not

reduce basal levels of CIITA in kidney or class II staining in renal interstitial cells, but does reduce the ability of rIFN-γ to induce CIITA and class II expression in kidney (16). To address the role of IRF-1 in the activation of each CIITA promoter *in vivo*, we injected WT and IRF-1KO mice with rIFN-γ and harvested their kidneys 4 hours later, at the peak of CIITA mRNA expression in kidney (see Figure 6.3). The rIFN-γ induced PIV mRNA and total CIITA mRNA in kidneys of both WT and IRF-1KO mice, but the induction was reduced in the IRF-1KO mice (Figure 6.5). This induction presumably coincides with the class II induction seen in tubular epithelial cells (16). The rIFN-γ failed to induce either PI or PIII (Figure 3C). Thus the role of IRF-1 in CIITA mRNA induction may be attributed partially to effects on PIV.

Two peaks of CIITA mRNA expression and PIV activation after a single LPS Injection. LPS strongly induces class II mRNA and class II product expression in the kidney epithelium by day 4 (12;14). BALB/c mice were given LPS and their kidneys were harvested at various times (Figure 6.6). Neither PI nor PIII were induced above the baseline at any time. PIV and total CIITA mRNA expression displayed two peaks of approximately 10-fold induction at 12 and 96 hours after a single LPS treatment. Thus the principal effect of LPS is on the IFN-γ-inducible promoter PIV.

Similar to rIFN-γ injection, LPS upregulates class II expression on the tubular epithelium in kidney. I studied whether LPS also had effects on the interstitial cell population. I counted interstitial cells stained by

immunohistochemistry and radiolabelled total kidney homogenate separately for their class II product expression (Figure 6.7). Twenty-four hours after LPS injection and 12 hours after maximal CIITA expression, the number of class II positive interstitial cells identified by immunostaining declined (Figure 6.7A). Total renal class II levels in this experiment were higher than sham injected mice by day 2 and remained at high levels through the remainder of the experiment (Figure 6.7B), similar to the changes observed after rIFN-γ injection. Thus the late CIITA expression at 96 hours in response to LPS could be due to changes in class II positive cell populations including trafficking, induction, maturation and/or loss of class II expression.

I examined whether the increase in CIITA and PIV mRNA by LPS were IFN-γ-dependent. We injected WT and GKO mice with LPS and harvested the kidneys at 12 and 96 hours, the peaks of total CIITA mRNA (Figure 6.8). PIV and total CIITA mRNA was increased approximately 10-fold in WT mice in response to LPS injection at both 12 and 96h, but not in the GKO mice at 12 h (Figure 6.8A) nor 96 h (Figure 6.8B). In response to LPS, PI was not activated at 12 hours and, by 96 hours, there were small changes in PI that were similar in GKO and WT mice. Thus the principal effect of LPS on the CIITA promoters is IFN-γ-dependent induction of PIV mRNA levels.

Induction of CIITA promoters in the kidney by allogeneic stimulation. Graft rejection at a remote site strongly induces class II expression in kidney via the systemic effects of IFN-γ (17). We have previously shown that the rejection

of the allogeneic ascites tumour cells (P815) in the peritoneal cavity induces massive class II expression on kidney tubular epithelium and vascular endothelium by day 7, mediated by IFN-γ and IRF-1 (12;16;18). I therefore examined whether CIITA promoters were activated in response to allogeneic stimulation and if IFN-γ and/or IRF-1 were required. WT, GKO, and IRF-1 KO mice were injected with P815 cells on day 0 and their tissues were harvested on day 7. PI showed mRNA induction (approximately 2-fold) in WT and GKO mice (Figure 6.9). Allogeneic stimulation induced total CIITA mRNA expression and PIV activation approximately 10-fold in the kidney from WT mice but not from the GKO mice, consistent with previous observations (12;14). The induction of total CIITA mRNA expression and PIV usage was somewhat reduced in IRF-1 KO mice compared to WT mice, indicating a role for IRF-1 in PIV and total CIITA mRNA induction (Figure 6.10). Thus PIV usage by allostimulation is completely dependent on IFN-γ and partly dependent on IRF-1.

Activation of PIV by a non-IFN-γ mechanism in injured kidney. The expression of class II and CIITA is induced by IFN-γ-dependent and IFN-γ-independent mechanisms in injured kidneys (12;19). I examined the response of each promoter to local injury. Briefly, the left kidney was cross-clamped to induce ischemic acute tubular necrosis (ATN) with the uninjured right kidney serving as the control. When the kidneys were examined 7 days later, the injured left kidney showed increased expression of CIITA mRNA and of mRNA from all three promoters, both in WT mice and in GKO mice (Figure 6.11),

consistent with the previously reported increases in class II products (12;20). Of interest, ATN in GKO mice induced increases in PIV usage, albeit to a lesser extent than in WT mice. Thus the IFN-γ-inducible promoter PIV is activated by both IFN-γ-dependent and non-IFN-γ mechanisms in response to local injury.

V. DISCUSSION

Not only does this report confirm that the behaviour of the CIITA promoters *in vivo* is consistent with the roles previously suggested for those promoters by studies in cell lines, it also points to additional levels of control. The changes in mRNA levels in these studies do not necessarily reflect altered transcription, and in some cases may reflect interstitial cell population changes. I found that the relative levels of promoter-specific mRNA in mouse tissues varied with the tissue and the stimulus. In the basal state spleen expressed significant levels of mRNA from all three promoters, while kidney expressed mainly PI and some PIV (about 4-fold less than PI). Overall, the basal levels of PI and PIV mRNA from were both IFN-γ-independent and IRF-1-independent. Total CIITA mRNA expression in the kidney mirrored PI expression in the basal state and PIV expression in the induced states. Treatment with rIFN-γ or stimuli that induced endogenous IFN-γ production induced high levels of PIV mRNA. Consistent with its role as the B cell specific promoter, PIII showed very low expression in kidney and was not induced by inflammatory stimuli. There were other levels of control *in vivo* that were not anticipated: the increased PIII mRNA in kidneys of IFN-γ

deficient mice; the independence of PIV mRNA from IFN-γ and IRF-1 in the basal state; the effect of rIFN-γ on PI expression; and the ability of kidney injury to increase PIV mRNA levels without IFN-γ.

The basal expression of PI in non-lymphoid organs was mainly independent of IFN-γ and IRF-1 and, surprisingly, PIV mRNA was also independent of IFN-γ and IRF-1. Basal class II expression in non-lymphoid tissues in mice is in the interstitial cell population and the present observations are consistent with a major role for PI in the basal levels of CIITA and class II. By immunohistochemistry, class II staining in mouse kidney is confined to interstitial cells in the basal state and is largely IFN-γ - and IRF-1 -independent (12;21). But class I and class II molecule expression is partially dependent on basal IFN-γ production, at least in arterial endothelium (12;20;22), indicating that many tissues are exposed to low levels of IFN-γ in the basal state. Thus PIV mRNA in the basal state might be expected to be IFN-γ - and IRF-1- dependent. The finding of IFN-γ- and IRF-1 independent expression of PIV is a surprise and suggests the existence of additional pathways for the control of PIV. This conclusion is supported by our demonstration of IFN-γ- independent increases in PIV mRNA after tissue injury. It is possible that other species will differ from mice in their use of the CIITA promoters in non-lymphoid organs, particularly in the basal state. While mouse endothelial cells and tubular epithelial cells do not show basal class II immunostaining, some humans and some rat strains (23) express class II in epithelial and endothelial cells as well as in interstitial cells (24). It is reasonable to expect that basal class II expression in renal interstitial

cells will reflect PI usage. The interstitial cells lack the markers of mature dendritic cells and are anti-DEC 205, -BB1/B7 and -B7-2 negative (data not shown) (25;26). True dendritic cells are very rare in normal kidney in the basal or induced states (27;28). However, PIV may prove to be the promoter that mediates the induction of CIITA and thus of class II in other resident renal cell types, through basal IFN-γ or by other mechanisms. Some interstitial cells could also use PIV in the basal state and for responses to environmental signals.

PIV emerges as the vital point at which CIITA and thus the class II system is regulated in disease states. The massive class II expression (i.e. in the kidney tubules) which is induced by systemic inflammatory stimuli *in vivo* is completely dependent on IFN-γ (12;16). From data presented here and previous time course experiments (14), this class II increase may be mediated primarily through PIV of CIITA. GKO mice showed no induction of the net CIITA mRNA expression or PIV activation, indicating that PIV usage in WT mice is due to IFN-γ production. Comparing WT, GKO and IRF-1KO mice, PI was not activated above controls in response to LPS or rIFN-γ injection at peak CIITA levels. Thus, in the IFN-γ induced states, the total CIITA mRNA expression in the kidney correlates mainly with the activation of PIV. The present results also provide evidence that some of the PIV induction *in vivo* depends on IRF-1, consistent with our previous demonstration that CIITA mRNA and class II protein expression were partially IRF-1 dependent (16;18). The persistence of some induction in the IRF-1KO mice may reflect the effects of the STAT1α -binding site in PIV, but other IRF

family members could also play a role. PIV contains IRF-1 and STAT-1 binding sites *in vitro*, although the IRF site may be dominant (8;29).

Our injury model demonstrates that PIV as well as PI and PIII mRNAs can be increased in tissues by non-IFN-γ mechanisms *in vivo*. We previously showed that acute renal ischemic injury induced the expression of MHC class II and CIITA. This induction was reduced in both GKO and IRF-1KO mice, but there were components of the induction that were not IFN-γ dependent (12;19). The present results demonstrate that, in both WT and GKO mice, all three CIITA promoters were activated in injured kidneys, demonstrating that injury induces the IFN-γ-inducible PIV with non-IFN-γ stimuli. The increase in mRNA could reflect increases in class II expression by injured epithelial cells, since class II staining is induced in the epithelium in the days following injury. It could also reflect accumulation of class II positive interstitial cells which occurs several days after renal injury (12). In GKO mice, there are as many class II positive interstitial cells after injury as in WT mice (12). Compared to the MHC induction that accompanies allogeneic stimulation or rIFN-γ (10-fold), these responses are weak (approximately 2-fold) (12). Factors involved in the activation of CIITA promoters in the injured kidneys are not known because the molecular mechanisms through which ischemia leads to tissue damage have not yet been defined. However, the MAP kinase family and the induction of transcription factors have been implicated in tissue injury following ischemia in the heart (30).

Our results presented a number of observations consistent with additional levels of CIITA control which had not been previously documented, i.e. the

inhibitory effect of IFN-γ on PIII mRNA levels in the basal state as indicated by the high levels of PIII in GKO mice. These high levels in the basal state are consistent with an IFN-γ dependent repressor for PIII and/or with population changes. Constitutive positive regulatory sites have been described for PIII in B cells, and the binding factors are independent of IFN-γ (31). Further, IRF-1 is not involved in the basal expression of PIII (Table 1). IFN-γ-dependent and/or IRF-1-independent repressors have been identified (5;32-34) and here a putative repressor could be ICSBP since there are potential ISRE sites within in the PIII promoter (5). Since the ICSBP promoter has a GAS site, ICSBP can act independent of IRF-1 as an IFN-γ-inducible repressor for PIII whereas all ICSBP would be lost in the GKO mice, in agreement with our data. Population changes could also account for PIII changes in GKO mice. However, there have been no published differences in infiltrating cells or in renal cell pathology in the basal state in the GKO mice; but some population differences may be difficult to detect. I favour the idea of a combination of effects; an IFN-γ-dependent repressor in the basal state cannot be ruled out.

Although we tend to consider changes in mRNA expression as reflecting events within cells (i.e. transcriptional effect), the changes in PI mRNA after IFN-γ could be a cell population change, potentially related to the ability of IFN-γ to regulate expression of certain chemokines (35;36). One difference between studies of class II expression *in vivo* and *in vitro* is the potential for cell population variance *in vivo* with respect to timing and sensitivities, which may affect observed promoter-specific mRNA levels. The changes in class II expression in a

complex tissue such as kidney reflect a number of distinct events. The increase in class II expression in tubular epithelial cells represents a change in expression at the single-cell level. However, the interstitial cells show variations in class II either due to altered cell numbers or altered expression per cell, which may have multiple relationships with and dependency on IFN-γ and/or IRF-1. The changes in the behaviour of class II-expressing interstitial cells could reflect the PI and PIII results in multiple cell-types depending on the context of various stimuli.

The use of multiple promoters of different responsiveness in the CIITA gene permits great flexibility in regulating the production of transcripts in relationship to the many roles of class II in many cell types. Through its role in class II induction, CIITA is critical to CD4 T cell differentiation, maintenance, surveillance, and effector functions in many tissues and therefore must be inducible by tissue-specific and stimulus-specific factors. Three major patterns of class II expression can be identified - the dendritic cell pattern, the B cell pattern, and the inducible pattern in tissues, and each is controlled by a different CIITA promoter. Multiple promoters demonstrating tissue specificity and differential expression have been described in other genes, i.e. insulin-like growth factor II (37), growth hormone receptor (38) and interleukin 5 receptor α subunit (39). However, the majority of studies have not addressed the complexity of the issue of tissue-specific and stimulus-specific promoter usage *in vivo*. Previous studies of CIITA identified various unique sequences of promoters for both human and mouse genes and showed only *in vitro* activity of the promoters (4-6;8). Our results show that the three mouse CIITA promoters are differentially activated in

various immune-induced states and tissues. The regulation of CIITA reflects class II expression in various tissues and disease states; as well, CIITA likely integrates information from various sources to impact antigen presentation depending on cell type or stimulus. Thus CIITA remains not only an excellent target for immune modulation during tissue-specific disease processes but also an instructive example of how to differentially regulate a vital gene in relationship to its different roles.

VI. TABLES

Table 6.1: Posphor Imaging data from various experiments shown in Figures 1-5 and from repeat experiments not shown. All data are generated from using the internal common exon 2 probe. Units are arbitrary, but conditions are the same within each experiment depictd in each row.

PI		PIII		PIV	
Balb/c	**GKO**	**Balb/c**	**GKO**	**Balb/c**	**GKO**
39	43	2	19	1	1
15	19	1	13	0.5	0.1
28	45	2	6	1	0.4
33	47	3	6	1	0.4
43	28	2	5	6	5
54	24	3	9	5.6	5.6
129	**IRF-1 KO**	**129**	**IRF-1 KO**	**129**	**IRF-1 KO**
94	25	14	18	0.8	0.4
78	22	12	11	2	1
26	17	5	3	6	1
127	87	35	21	26	24

Table 6.2: Ratios and means of Phosphor Imaging data for WT:GKO and WT:IRF-1 KO for each promoter from kidneys in their basal states*. Various experiments including data not shown are summarised, all experiments performed using the common exon 2 probe under identical conditions

	WT:GKO*			WT:IRF-1 KO**			
	PI	PIII	PIV	PI	PIII	PIV	
	0.9	0.1	1	3.8	0.8	2	
	0.8	0.07	5	3.6	1.1	2	
	0.6	0.3	2.5	1.5	1.7	6	
	0.7	0.5	2.5	1.4	1.7	1.1	
	1.5	0.4	1.2				
	2.25	0.3	1				
mean^	1.1	0.3	2.2	2.6	1.3	2.8	mean^
P(T<=t) ^^	0.7	0.0001	0.1	0.09	0.3	0.2	P(T<=t) ^^

* Ratios of Phosphor Imaging data for BALB/c:GKO mice from Table 1

**Ratios of Phosphor Imaging data for 129/J:IRF-1 KO mice from Table 1

^ Ratios of Phosphor Imaging data for WT/KO mice are compared to one (no variance between groups).

^^ A two-tailed t-Test was performed on the means.

VII. FIGURES

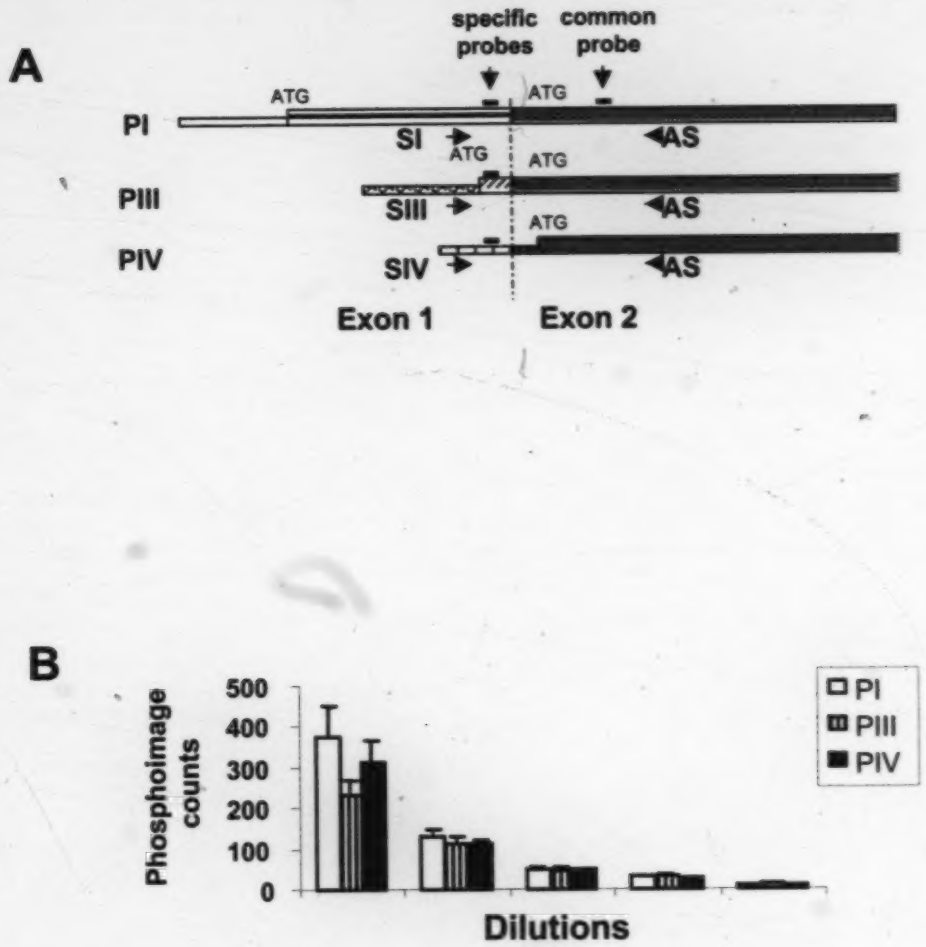

Figure 6.1: RT-PCR and cloning strategy for CIITA promoter-associated 5' UTRs. (A) Common antisense primers and specific sense primers were used to amplify each CIITA promoter-associated 5' UTR. PCR products were quantitated using a common oligonucleotide probe. (B) PCR products were cloned, quantitated and PCR-amplified. An approximate standard 3-fold dilution curve was made. The Y-axis depicts arbitrary Phosphor Imaging counts. These data are representative of 10 determinations.

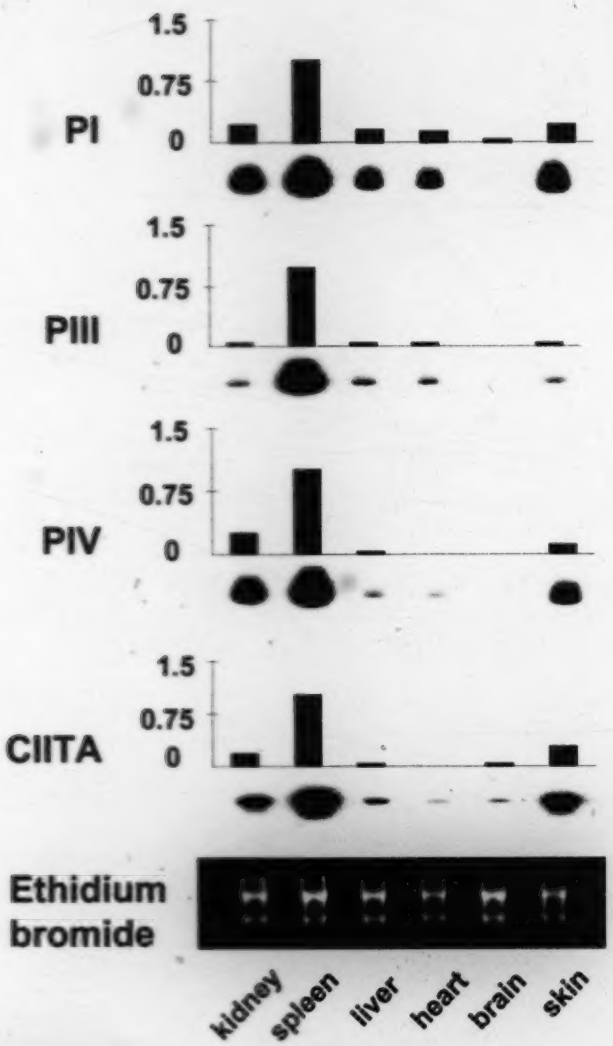

Figure 6.2: Relative expression of CIITA promoters in mouse tissues in the normal state. Tissues from five 129/J mice were harvested and total RNA was extracted by the guanidinium-cesium chloride method as described. Total CIITA mRNA and 5'-UTRs of each CIITA exon 1 mRNA were amplified by RT-PCR (30 cycles), Southern blotted, and probed with human CIITA cDNA and mouse cDNA for 5'-UTRs of each CIITA exon 2, respectively. The blots were handled identically and quantitated by Phosphor Image analysis. The Y-axes depict arbitrary Phosphor Imaging counts

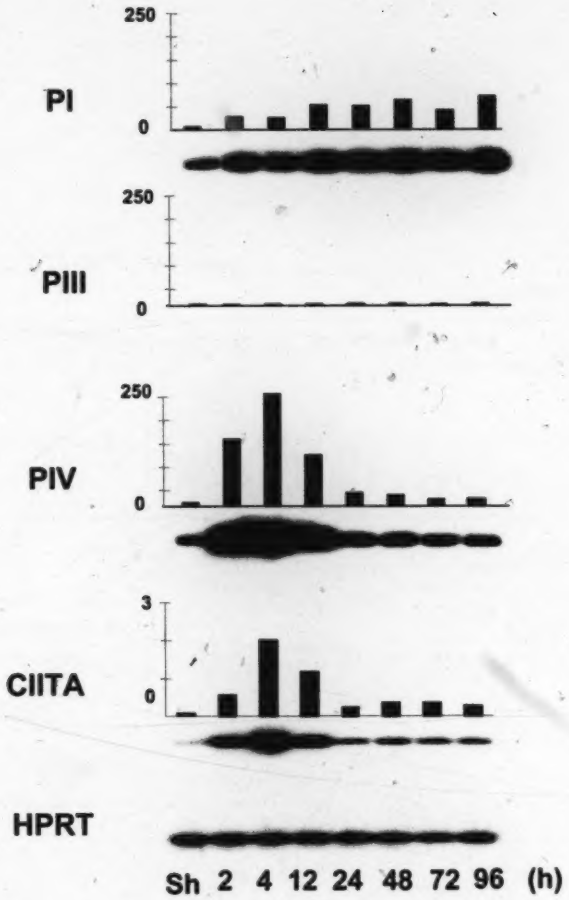

Figure 6.3: rIFN-γ administration induces promoter usage in vivo in kidney. Five BALB/c mice in each group were injected with 100,000 U of rIFN-γ and their kidneys were harvested after various times. Total RNA was extracted as described. Total CIITA mRNA and 5'-UTRs of each CIITA mRNA were amplified by RT-PCR (30 cycles), Southern blotted, and probed with total CIITA cDNA and oligonucleotides specific for exon 2, respectively. The blots were handled identically, quantitated by Phosphor Image analysis, and monitored for RNA content by HPRT Phosphor Imager counts. The Y-axes depict arbitrary Phosphor Imaging counts

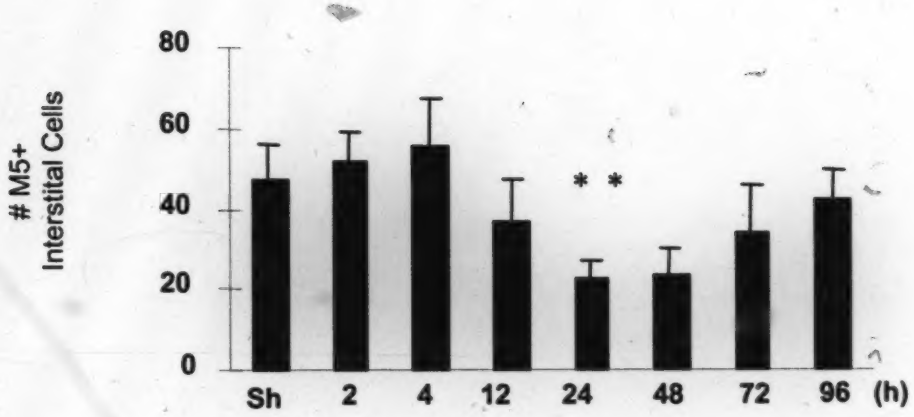

Figure 6.4: rIFN-γ administration induces changes in class II expression in kidney. BALB/c mice were injected with 100,000 U of rIFN-γ and their kidneys were harvested after various times. Class II positive interstitial cells were counted in each of five fields and the total was averaged. *ANOVA $p<0.001$.

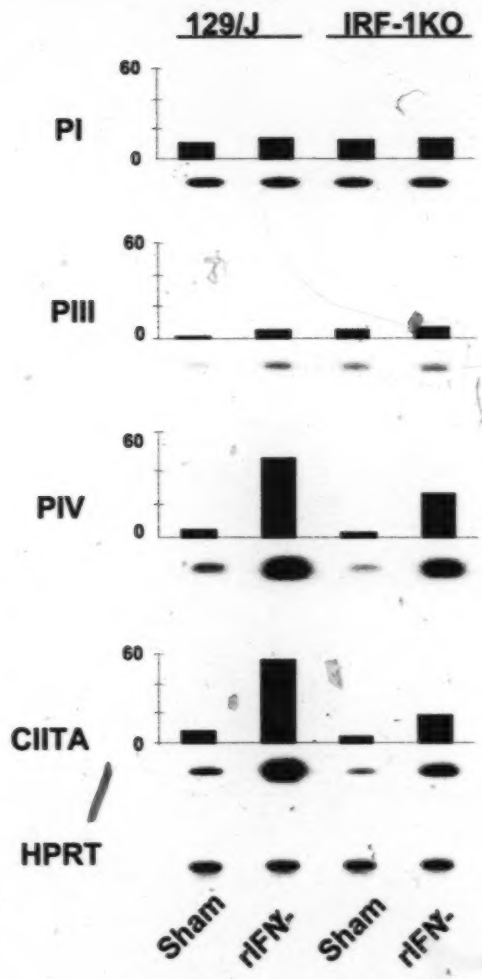

Figure 6.5: rIFN-γ administration induces changes in IRF-1-dependent PIV promoter activation in kidney. Five 129/J and IRF-1 KO mice were injected with 100,000 U of rIFN-γ and their kidneys were harvested after 4 hours. Total RNA was extracted as described. Total CIITA mRNA and 5'-UTRs of each CIITA mRNA were amplified by RT-PCR (30 cycles), Southern blotted, and probed with total CIITA cDNA and oligonucleotides specific for exon 2, respectively. The blots were handled identically, quantitated by Phosphor Image analysis, and monitored for RNA content by HPRT Phosphor Imager counts. The Y-axes depict arbitrary Phosphor Imaging counts

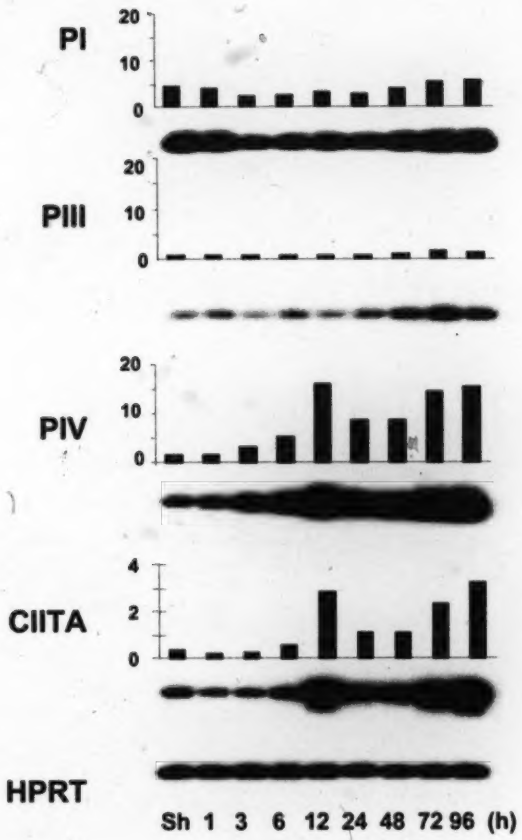

Figure 6.6: A single LPS administration induced dual peaks of CIITA mRNA expression in the kidney. BALB/c mice were injected with LPS (25 μg) and were harvested at various time points. Total CIITA mRNA and 5'-UTRs of each CIITA mRNA were amplified by RT-PCR (30 cycles), Southern blotted, and probed with CIITA cDNA or oligonucleotides specific for exon 2, respectively. The blots were quantitated by Phosphor Image analysis, and monitored for RNA content by HPRT Phosphor Imaging counts. The Y-axes depict arbitrary Phosphor Imaging counts

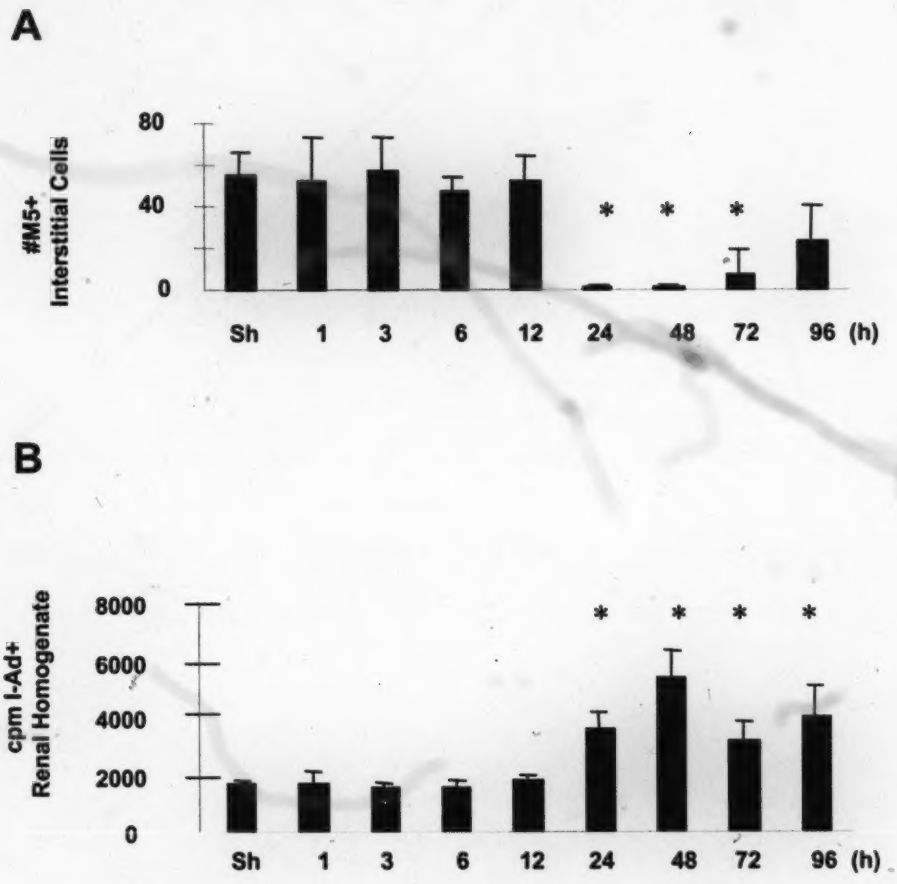

Figure 6.7: A single LPS administration induced changes in class II expression in the kidney. BALB/c mice were injected with LPS (25 μg) and were harvested at various time points. (A) Class II positive interstitial cells were counted in each of five fields and the total was averaged. (B) Class II RABA was done on renal homogenates and cpm measured. *ANOVA $p<0.01$

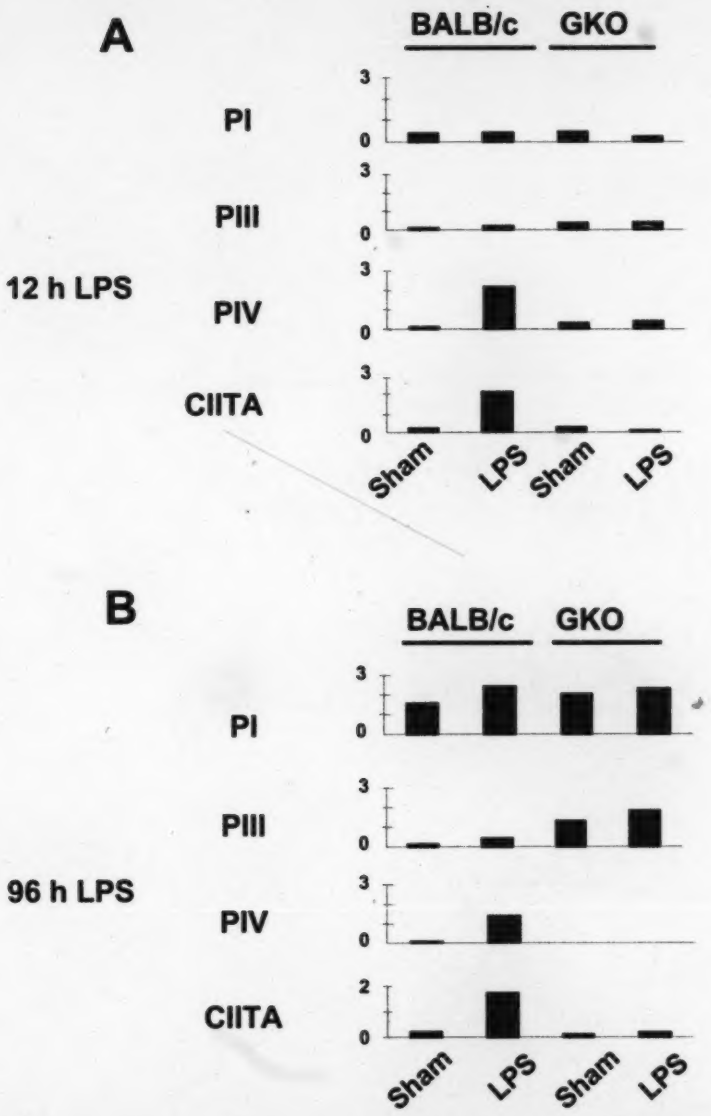

Figure 6.8: A single LPS administration induced dual peaks of CIITA mRNA expression and IRF-1-dependent PIV activation in the kidney. BALB/c mice were injected with LPS (25 μg) and were harvested at 12 and 96 hours. Total CIITA mRNA and 5'-UTRs of each CIITA mRNA were amplified by RT-PCR (30 cycles), Southern blotted, and probed with CIITA cDNA or oligonucleotides specific for exon 2, respectively. The blots were quantitated by Phosphor Image analysis, and monitored for RNA content by HPRT Phosphor Imaging counts. The Y-axes depict arbitrary Phosphor Imaging counts. The data are representative of at least two separate experiments.

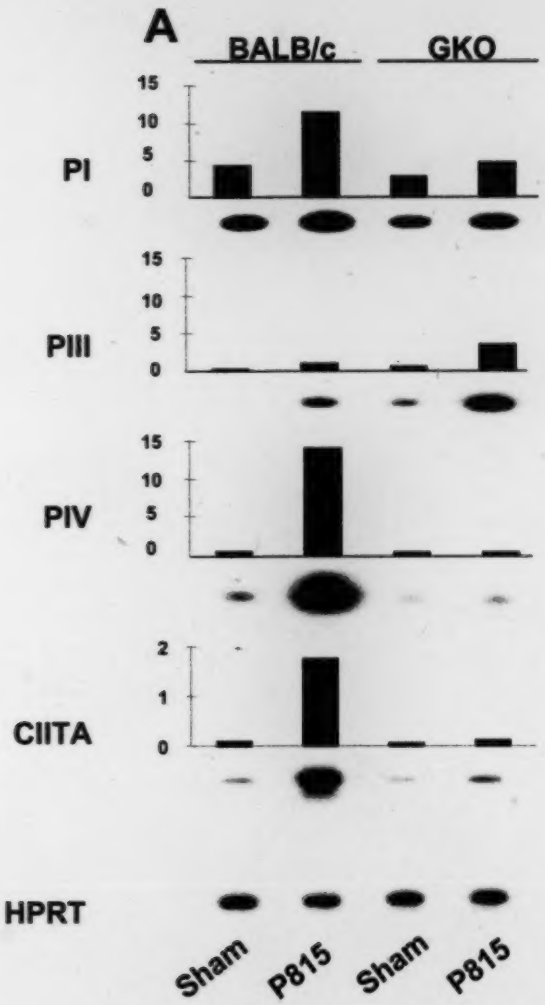

Figure 6.9: Induction of CIITA promoter activation in the kidney after allogeneic stimulation. BALB/c and GKO mice were injected with 20 x 10^6 P815 cells on day 0 and their kidneys were harvested on day 7. Total CIITA mRNA and 5'-UTRs of each CIITA exon 1 mRNA were amplified by RT-PCR (30 cycles), Southern blotted, and probed with CIITA cDNA and oligonucleotides specific for exon 2, respectively. The blots were quantitated by Phosphor Image analysis, and monitored for RNA content by HPRT Phosphor Imaging counts. The Y-axes depict arbitrary Phosphor Imaging counts

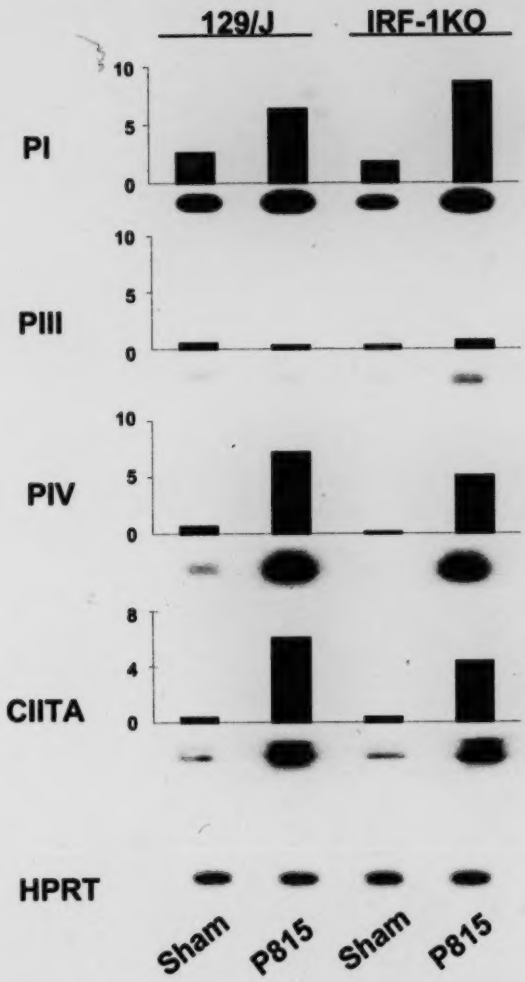

Figure 6.10: Induction of CIITA promoter activation in the kidney after allogeneic stimulation.129/J and IRF-1 KO mice were injected with 20 x 10^6 P815 cells on day 0 and their kidneys were harvested on day 7. Total CIITA mRNA and 5'-UTRs of each CIITA exon 1 mRNA were amplified by RT-PCR (30 cycles), Southern blotted, and probed with CIITA cDNA and oligonucleotides specific for exon 2, respectively. The blots were quantitated by Phosphor Image analysis, and monitored for RNA content by HPRT Phosphor Imaging counts. The Y-axes depict arbitrary Phosphor Imaging counts

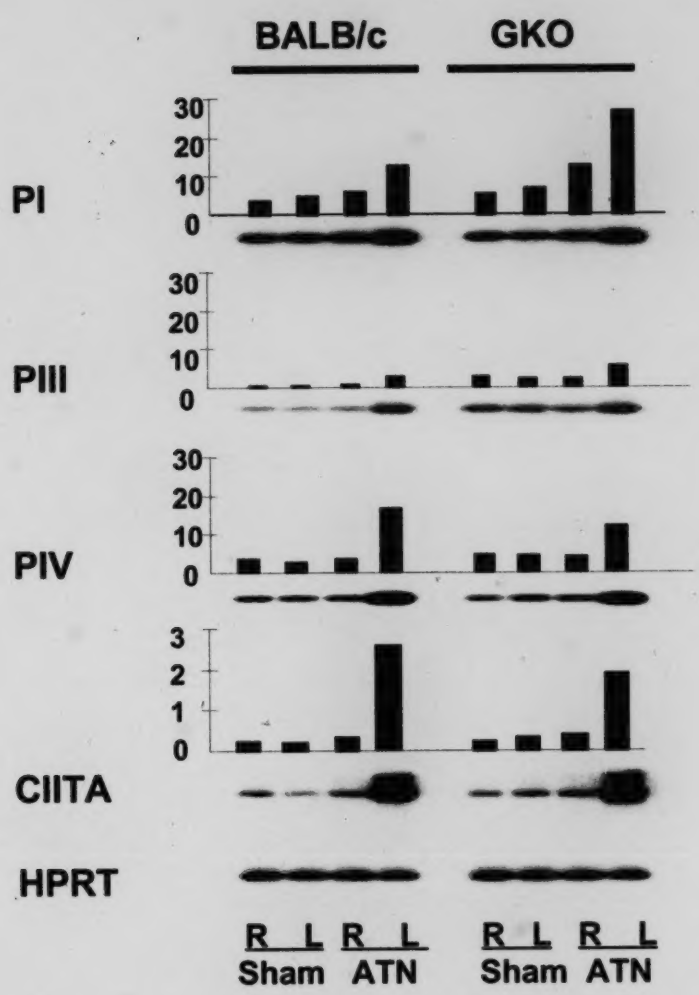

Figure 6.11: Activation of the three CIITA promoters in injured kidneys. Ischemic injury to the kidney was produced by clamping the left (L) renal pedicle for 60 min and was compared with the unclamped right (R) kidney. Sham-operated control mice underwent a simple laparotomy under identical conditions. The kidneys were harvested on day 7. Total CIITA mRNA and 5'-UTRs of each CIITA exon 1 mRNA were amplified under similar conditions by RT-PCR (30 cycles), Southern blotted, and probed with CIITA cDNA and oligonucleotides specific for exon 1, respectively. The blots were quantitated by Phosphor Imager analysis and monitored for RNA loading by HPRT Phosphor Imaging counts. The Y-axes depict arbitrary Phosphor Imaging counts.

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CHAPTER 7

General Discussion

Chapter 7

General Discussion

I. PREFACE

Working with the IFN-γ gene knockout mice gave me the opportunity to determine the roles of IFN-γ in MHC regulation *in vivo*. Early work showed that IFN-γ was present during MHC induction. Injecting anti-IFN-γ monoclonal antibodies indicated that IFN-γ was involved in MHC induction. Our work with the knockout mice demonstrated that IFN-γ is differentially responsible for MHC induction to varying degrees depending on the stimulus. Further I was able to describe the role of IFN-γ in the induction of MHC expression in various immune states. These experiments were exciting since they enabled us to define immunity invoked in the kidney, starting with MHC regulation. I went on to investigate MHC expression utilising the GKO, the IFN-γ receptor KO, IRF-1 KO and CIITAKO mice. I attempted to establish the pathways involved in MHC regulation in non-lymphoid tissues in the basal state and during inflammation, tissue injury and transplant rejection.

The specific role of MHC expression in a tissue is unclear. Perhaps the role of MHC at low levels is tolerogenic since no inflammatory stimulus is present to upregulate "signal two" through CD28/B7 interactions. The thought is that naïve T cell passing by MHC in the tissue would be anergised due to the

presentation of self antigen in the absence of costimulation. This has yet to be proven. The non-lymphoid tissue is very sensitive to IFN-γ levels and upregulates MHC expression in the epithelium in response to injury and inflammation. The picture emerges of a dynamic pattern of MHC changes not just in disease states but in day-to-day encounters with tissue injury and infections. The events in the non-lymphoid organs include altered gene expression in the epithelium and endothelial cells as well as altered populations of interstitial cells.

Although MHC molecule changes correlate with many disease processes, the causal role of regulated product changes during immune events in the tissues is difficult to resolve. Class II induction by IFN-γ in tubular epithelial cells confers some APC-like properties, as shown by their ability to take up and present foreign and self antigen *in vitro* and to stimulate hybridomas (1). For example, autoimmune nephritis in mouse models is promoted by IFN-γ but whether MHC induction is the mechanism of this effect is not clear (2;3). The lack of co-stimulator expression (i.e. B7) by renal epithelial cells may limit their ability to trigger primary responses and may render class II on kidney epithelium tolerogenic, although this area is controversial (1;4-7). It remains likely that the immune reactions in non-lymphoid organs are triggered primarily by interstitial cells (and possibly endothelium) but that MHC molecule expression in parenchymal cells is used for surveillance of infected cells at the effector level. In transplantation this is further complicated by the powerful alloantibody response against MHC antigens, which can destroy endothelium and perhaps parenchymal cells that express MHC. Thus in transplants, MHC can be the

priming antigen (by direct or indirect routes), the target of effector T cells, the target of alloantibody, as well as the tolerizing antigen.

MHC regulation in mouse kidney

Our laboratory studies MHC regulation *in vivo* in non-lymphoid tissues as a possible regulator of immune recognition during organ-specific disease states and graft rejection. We use the mouse kidney as a model because of its relevance to human kidney transplantation and kidney disease, and because the kidney has little contamination from other tissues (i.e. lymphoid tissue). We use a variety of gene-disrupted mice (including mice lacking IFN-γ, IFN-γ receptor α-chains, CIITA, or the transcription factor IRF-1) to examine the roles of IFN-γ and its signalling pathways in MHC gene regulation *in vivo*. We have developed a battery of stimuli that alter renal MHC expression: rejection of a kidney allograft, rejection of a P815 ascites tumour allograft, oxazalone-induced skin inflammation, and bacterial lipopolysaccharide (8-10). In addition, we induce renal tissue injury by unilateral ischemia (produced by cross-clamping the vascular pedicle of the right kidney for 60 minutes to produce reversible acute tubular necrosis) and by administering nephrotoxins (11-15). Using these stimuli, we have identified in mouse kidneys three states of class II expression: basal, systemically induced, and local injury induced. Changes in class I or class II expression can be mediated by altered gene expression and/or by population changes such as migration or cell maturation and division, underscoring the complexity of studying MHC regulation *in vivo*.

II. MHC regulation *in vivo*

It was thought that MHC class I was constitutively expressed *in vivo*. The rules have been changed to include IFN-γ as an inducer of class I, even in the basal state, leaving us with the question: what is "constitutive" *in vivo*? It is of interest that even in a mouse in the animal colony, basal levels of IFN-γ contribute to class I expression in remote tissues such as the kidney. The reasons for basal IFN-γ induction of class I on the arterial endothelium are unknown but could include tonic stimulation of naïve T cells as they traffic through the periphery since these interactions are known to be important for T cell survival (16-18).

It may be surprising but reassuring to those who study class II expression *in vitro* that class II expression in kidney follows many of the same rules as first revealed in cell lines. What is remarkable is that some of these changes are both as rapid and as intense as changes demonstrated *in vitro*. Renal class II expression is a prototype of class II expression in non-lymphoid tissues, and reflects a composite pattern of changes in gene expression, in cell populations and in maturation.

In vivo regulation of class I and II expression is a readily demonstrable, consistent, and a quantitatively impressive accompaniment to inflammation and tissue injury. As mechanisms of regulation are unravelled *in vitro* and *in vivo*, we need to focus more attention on experimental situations where the significance of

class II regulation in immune responses and disease states *in vivo* can be established.

A. The Basal State

MHC class I. We have demonstrated that IFN-γ is required for MHC class I expression on the arterial endothelium and in the glomeruli. Without IFN-γ, there is an approximate 40% reduction in total class I as measured in tissue homogenates. From staining tissue sections, the reduction is mainly from the arterial endothelium. It is difficult to know from our studies whether there is any MHC expression without IFN-γ, since we do not see staining of all tissues in the wild-type mice in the basal state. It is generally thought that all nucleated cells express class I in the basal state. I can say that the basal class I is reduced without IFN-γ, but that there is likely a component of class I expression that is independent of IFN-γ.

I found that class I is induced in the basal state by IFN-γ specifically in the arterial endothelium, since the immunohistochemistry staining was lost in the GKO mice. It is unlikely that this loss is a result of phenomena related to effects of IFN-γ gene disruption *per se* since the IRF-1 and the IFN-γ receptor gene-disrupted mice also show the impairment in class I on the arterial endothelium (9;19;20). Recent work in a xenograft model also showed that class I expression in the basal state on the arterial endothelium requires species-specific IFN-γ (21). This may reflect local IFN-γ effects in the kidney or a systemic release of IFN-γ

even in the basal state. The effect could be local since in a transplant model, neutralising the systemic IFN-γ with mAb (R4-6A2) as measured by MHC induction in the contralateral kidney does little to abrogate MHC expression in the transplant (unpublished data). The source of the IFN-γ is unknown in the basal state but could be NK cells since they produce IFN-γ without the need for activation. Whether the kidney tubules or other resident cells of the kidney produce IFN-γ is unknown, although some studies have pointed to IFN-γ inducing its own expression at remote sites such as the kidney (22).

In the kidney, the mRNA levels of the antigen presenting machinery are low and do not seem to be dependent on IFN-γ pathways. This was not proven here, however, since I could not measure the protein levels of each molecule in the kidney. However, basal mRNA levels of LMP7, TAP1 or H-2Mα were not noticeably reduced in the kidneys of IFN-γ gene-disrupted mice.

Although CIITA has recently been implicated in class I expression, I saw no evidence of a class I deficiency in CIITAKO mice in the basal state. To the contrary, the CIITAKO mice showed diffuse staining on their renal tubules in the basal state. This indicates a higher overall level of class I expression in the basal state in the absence of CIITA. This could reflect various mechanisms including higher overall IFN-γ levels in the basal state although others have (23) demonstrated levels of IFN-γ levels are not higher in these animals in the tissues they studied. These mice have higher IL-4 production, but the role of IL-4 in class I induction is not known (23).

MHC class II. In the basal state in humans, class II is expressed in the lymphoid organs but is also found in non-lymphoid organs on some endothelial and epithelial cells and on interstitial cells (24;25), which is a population that may include macrophages and dendritic cells. Human kidney expresses class II in the interstitial cells with low but variable expression on the tubules, vascular endothelium, and glomeruli (24;26). In kidney in the basal state, mice do not express class II on parenchymal or vascular endothelial cells (27); only interstitial cells express class II (28). This interstitial cell population may include sentinels surveying the environment for infection, damage or stress.

Basal class II expression in kidney (i.e. expression on renal interstitial cells) is normal in germ-free mice but is reduced by lethal irradiation, in keeping with its dependency on marrow derived cells (29). T cell deficient nude and SCID mice have normal or increased MHC expression in kidney, which is readily increased by environmental stimuli. There is no statistically significant reduction in basal class II expression in kidneys of GKO mice (13), GRKO mice (20), or IRF-1 KO mice (8;9) compared to control mice. MHC class II was slightly reduced without IFN-γ or IRF-1; thus I wondered if class II was also dependent on IFN-γ in the basal state. Subsequently, Pober's group has shown that class II expression in the basal state does require IFN-γ (21) at least in the arterial endothelium of pigs and humans. These cell populations in mouse, at least by the limits of immunohistochemistry, do not show basal class II expression in the kidney. Nonetheless, the dependence of basal class II expression on IFN-γ cannot be ruled out.

From my studies in the CIITAKO mice, class II expression in the basal state is likely dependent on CIITA. These findings are corroborated by evidence in the human where the Group A bare lymphocyte syndrome (BLS) patients also lack CIITA and their lymphocytes do not express class II. Class II is expressed in the basal state on B cells and on antigen presenting cells such as dendritic cells. The renal interstitial population is heterogeneous and includes cells in the peritubular capillaries (i.e. blood and endothelial cells) as well as interstitial cells. It is unknown which cell types are involved in the class II expression seen in the basal state or the degree to which these cells show IFN-γ dependent MHC expression. Regardless, I have shown by immunohistochemistry that class II expression in this population is completely dependent on CIITA in the basal state.

CIITA PIII mRNA levels in the GKO mice were significantly higher than in the wild-type mice in the basal state, so our results are consistent with population changes in the GKO mice or an IFN-γ dependent repressor for PIII. The literature has several examples of IFN-γ-dependent repressors of transcription. One early report showed that B cells display reduced class II expression through B-cell stimulation factor 1 (BSF-1) in response to IFN-γ when IL-4 was present (30). Furthermore, in the basal state, PIII usage is not changed in the IRF-1 KO compared to the WT mice. This indicates that IRF-1 is not involved in the basal usage of PIII. Constitutive positive regulatory sites have been recently described for PIII activity, at least in B cells, and these factors are IFN-γ independent (31). This putative IFN-γ dependent repressor could be IRF-2 or ICSBP, which are

negative regulators induced by IFN-γ, since there are potential ISRE sites within in the PIII promoter (32). IRF-1 is important for ICSBP induction (33) but IRF-1 and ICSBP have GAS sites in their promoters, indicating they are both directly activated in response to IFN-γ. Thus in the IRF-1 KO mice, ICSBP could still be acting as a repressor for PIII, compatible with our data. ICSBP is expressed in resting B cells and activated T cells and macrophages (34), compatible with ICSBP expression in the interstitial cell population. IRF-2 is a long-lived transcriptional repressor that binds the ISRE (35) but it has also been shown to activate several genes including the PIV of CIITA (36). There is also the potential that the PIII-expressing renal population is greater in the basal state in GKO mice compared to wild-type mice. However, there have been no published differences the cellular infiltrate or in renal cell pathology in the basal state in the GKO mice. That I could detect the changes with RT-PCR is feasible given the sensitivity, but it remains that cell population differences may be difficult to detect visually.

Of particular interest, promoter IV does not require IFN-γ expression in the basal state. Although a role for IFN-γ cannot be ruled out since I saw decreased expression of PIV mRNA in the GKO mice, this difference was not significant.

Together, our data for class I and II suggest that IFN-γ contributes to basal MHC expression in remote tissues such as the kidney. Within the kidney there are cell population differences with respect to the dependence on IFN-γ for MHC induction. With respect to the interstitial population, class II expression is largely IFN-γ and IRF-1 independent. There are also IFN-γ-independent mechanisms that influence some class I expression.

Section Conclusions.

The surprising role of IFN-γ in renal MHC expression in the basal state has several implications. First, IFN-γ must be constantly present in sufficient quantities in unstimulated mice to induce genes in the arterial endothelium, raising the question of whether this is also a feature of human endothelium. However, some class I is expressed in the absence of IFN-γ or its receptor, either due to constitutive expression or to induction by non IFN-γ stimuli. Mouse class II is more difficult to induce by IFN-γ than is class I, and this may explain (as illustrated here in the response to rIFN-γ) why class I is induced in the basal state but class II is not. This may be relevant to the human kidney where class II is present on many endothelial cells *in vivo*. I suggest that class II expression in human endothelium but not mouse endothelium reflects either higher IFN-γ production in the basal state in humans or greater sensitivity of human class II genes to basal IFN-γ production. Since IFN-γ potentiates atherosclerosis in the apoE knockout and with recent findings in a xenotransplant model (37), the fact that basal IFN-γ production can affect gene expression in renal endothelium is relevant to renal atherosclerosis.

B. The Response To Inflammatory Stimuli

MHC expression is induced strongly in kidney by intense inflammation anywhere in the host in response to graft versus host disease, graft rejection,

skin sensitisation to oxazalone, or bacterial LPS. Potent local inflammation such as in response to oxazalone skin painting induces IFN-γ-dependent MHC expression systemically. This response to inflammation is highly dependent on IFN-γ, but may at times reflect synergy with other cytokines, particularly TNF-α (38). Likewise, intense MHC expression in a rejecting transplant is a systemic process; class I and II molecules are intensely induced in the host kidneys and other organs as well as in the transplanted organ (39).

MHC molecule induction by potent inflammatory stimuli is highly dependent on IFN-γ and reflects induction in epithelial and endothelial cells (9;10;13;40). Studies presented herein demonstrate that in response to systemic rIFN-γ or LPS administration, MHC induction in the kidney is almost completely dependent on IFN-γ. Other studies also demonstrate that systemic stimulation induces MHC in the kidney and that the induction is dependent on IFN-γ (9;13;20). For example, in response to P815 (DBA/2) tumour cells injected i.p., BALB/c mice develop ascites and splenomegaly at 7 days and then reject the tumour due to multiple minor histocompatibility antigen disparities. This response was shown to be T cell dependent (40-42). With P815, as in the experiments shown here with rIFN-γ and LPS injections, class I is massively induced in renal tubules, glomeruli, and arteries in wild-type mice, whereas the kidneys of GKO or IFN-γ Rα gene-disrupted (GRKO) mice show no staining. Similarly class II expression was strongly induced in tubules of wild-type mice but not in GKO or GRKO mice. IRF-1KO mice also showed reduced MHC induction in response to P815 stimulation. In wild-type mice, anti IFN-γ mAb prevented the induction of

IFN-γ mRNA and MHC expression. Thus the upregulation of MHC molecule expression in response to systemic inflammatory stimuli is highly IFN-γ dependent.

IFN-γ (20,000 to 100,000 units i.p.) is the only recombinant cytokine that strongly induces MHC molecules in renal epithelial cells and endothelial cells *in vivo*. MHC molecules on the renal tubular epithelium are likely expressed on the basolateral membrane since I see staining on the perimeter of the tubules, presumably for surveillance by T cells in the interstitium. During intense stimulation, class I and class II is also induced on the endothelium of arteries. TNF-α synergizes with IFN-γ to induce class II in renal epithelium, but does little without IFN-γ (43;44). No other cytokine that we tested induces MHC molecules in the parenchymal and endothelial cells of the kidney *in vivo*. In GKO mice, rIFN-γ induces renal MHC molecules as strongly as in WT mice, but GRKO mice have no response to rIFN-γ (20). However, there remained the potential for other mechanisms that could bypass the lack of IFN-γRs, particularly during complex inflammatory states where numerous cytokines with potential for MHC regulation (i.e. IFN-α/β or TNF-α) are produced. The induction of renal MHC class I and II expression by systemic inflammatory stimuli, which are potent inducers of many cytokines, was essentially lost in GRKO mice. This laboratory previously showed that IFN-γ could induce its own mRNA in kidney (45), presenting the possibility of self-amplification. Thus mice with intact IFN-γ genes could have stronger responses to rIFN-γ than do GKO mice. However, these studies show that this

does not occur, at least with single doses of rIFN-γ. However, the high MHC expression that occurs in response to systemic inflammation is dependent on IFN-γ. This does not imply that IFN-γ is the only cytokine operating because synergy with other cytokines increases the effect of IFN-γ on MHC expression (43;44). Nevertheless the high cytokine levels in these inflammatory states apparently cannot bypass the absence of IFN-γ or IFN-γRα.

Class I and II induction on the tubular epithelium is mediated by IRF-1, as demonstrated in the IRF-1 KO mice (8;9). IRF-1 is transcriptionally induced by IFN-γ and activates the transcription of target genes through sites in their promoters (ISRE, interferon-stimulated response element) (46). When mice are challenged with LPS, oxazalone, tumour allograft rejection, or injected rIFN-γ, the renal tubules of IRF-1KO mice show low class I and II molecule expression as assessed by immunoperoxidase staining of tissue sections (8;9). The lack of MHC class I and II induction in the IRF-1KO mice has been verified by RABA and mRNA assessments (8;9).

Class II induction is completely dependent on CIITA but only partly dependent on IRF-1. In response to systemic stimuli, the induction of class II is likely mediated by the PIV of CIITA and is largely dependent on IRF-1. This state is accompanied by poorly understood changes in PI expression and by changes in the interstitial cells' class II expression. The newly described PIII CIITA 5'-regulatory region (15;32;47) contains two potential GAS sites and up to 4 potential IRF-1 binding sites, as defined by the consensus sequence G(A)AAA G/C T/C GAAA G/C T/C (48;49). Furthermore, CIITA induction by rIFN-γ is

severely reduced in IRF-1 KO mice (9). Thus, the partial cycloheximide sensitivity of CIITA induction, the reduced response of IRF-1 KO mice, and the presence of IRF-1 sites in the CIITA promoter may reflect the requirement for both pre-existing STAT1α and newly synthesised IRF-1 for full expression of CIITA *in vivo*.

In response to systemic stimuli, the antigen presenting machinery is upregulated. In fact in every instance that I saw induction of MHC class I and II, the antigen presenting machinery is also upregulated, at least at the mRNA level. This is of particular interest because it is unknown whether the kidney can present antigen to stimulate T cells. Our experiments would suggest that the kidney mobilises the machinery to present antigen under inflammatory conditions. Furthermore CIITA also differentially uses its promoters in response to inflammatory stimuli in the kidney. The IFN-γ sensitive promoter-associated mRNA is upregulated in response to LPS and rIFN-γ. Thus our experiments support the idea that the kidney presents antigen and that it does so in response to inflammatory cues.

The differences between our results with LPS (class II induction was IFN-γ dependent) and those of Haas *et al.* (50) (class II induction independent of IFN-γ) probably reflect variations in experimental protocol. Haas *et al* studied MHC induction by staining tissues 4 days after injection of 50μg LPS from *E. coli*, noting that LPS induced MHC class II expression in renal tubules of GRKO mice. They concluded that IFN-γ is not absolutely required for induction of class II after LPS treatment. We found little or no class II induction by *S. minnesota* LPS in

GKO or GRKO tubules, compared to controls. One possible mechanism for the induction of class II by LPS in GRKO mice in the studies of Haas *et al.* is that they used an LPS protocol that induced some renal injury and thus led to the type of class II expression that follows acute tubular injury. We selected heat inactivated-LPS from *S. minnesota to* avoid the stress response and injury and thus the only effects I elicited were those related to IFN-γ production.

In addition to epithelial and endothelial induction of class II, inflammatory stimuli change the interstitial cell population. When mice are injected with rIFN-γ or LPS their kidneys show a decrease in class II staining in the interstitium (measured by counting class II stained interstitial cells in tissue sections) despite the massive induction of class II in epithelia and endothelia (8;13). While cessation of class II expression could explain the data, it is more likely that this change reflects migration of some of the interstitial cell population, since several chemokines are regulated by IFN-γ (51;52). Thus IFN-γ may have multiple effects on renal MHC expression including upregulation of class I and class II in the parenchymal cells and some endothelial cells, as well as mobilisation of cells.

Section conclusions

The regulation of MHC gene expression in kidney is becoming a major example of the complex yet precise mechanisms that regulate gene expression in response to local and remote influences. IFN-γ plays unique and non-redundant roles in the induction of both MHC molecules and the antigen presenting machinery mRNA levels. CIITA also plays a unique role in the class II

response to inflammation, but plays no role in the induction of class I after LPS or P815. CIITA in renal cells use predominantly PIV in response to systemic stimuli (Figure 7.1). This reflects class II expression on the tubules. These results have implications for autoimmune nephritis since the disease has been shown to be dependent on class II expression in the kidney, at least in one model (53).

C. The Local Injury Response.

Numerous pathways are implicated in the pathogenesis of renal injury. These include chemokines, cytokines, alloantibodies and the activation of coagulation and complement pathways and release of reactive oxygen species. (54) Beginning several days after renal injury, class I and II molecules are transiently induced in the tubular epithelium of the injured kidney as well as in the interstitial cell population (12). This induction is weaker than observed with systemic stimuli (about a 2-3-fold increase vs. 8-15-fold, respectively) (10;11;13;15). The feeling is that injurious factors such as hypertension and delayed graft function (ATN) play causative roles in transplant rejection, perhaps later on in the graft's life span. Early events such as acute rejection predispose the graft to a cycle of inflammation, injury and rejection episodes (55). In fact, cisplatin injury prior to transplantation in a MHC-mismatched mouse renal transplant model increases the pathophysiological lesions in the transplanted kidney compared to uninjured control kidneys (L-F Zhu, M. Afrouzian and PF. Halloran, unpublished).

The response to injury is not dependent on the source. We observe the same response in both ischemic or toxic acute tubular necrosis (ATN). In unilateral ischemic ATN, the contralateral kidney, serving as a control, shows neither injury nor enhanced MHC molecule production. Ischemic or toxic injury in GKO, GRKO, and IRF-1KO mice induces MHC expression but at a reduced level compared to WT mice (13;15;20). The mechanisms of MHC molecule induction in epithelium are not known. Such changes may be relevant to the ability of injury to alter the immunogenicity of the tissue in autoimmune disease and transplantation (56).

Acute renal injury in GKO, GRKO, and IRF-1 KO mice induced MHC class I and II expression locally in epithelial cells, showing unequivocally that mechanisms independent of IFN-γ can induce class I and II in epithelia. Injury in CIITAKO mice induced only class I expression, showing that CIITA is the molecule through which even the IFN-γ independent mechanisms in class II regulation must pass. Ischemic injury to mouse kidney induces an inflammatory response which we call the "injury response" in which MHC class I and II genes and many cytokine genes are expressed: IFN-γ, IL-2, GM-CSF, TGF-β1, TNF-α, IL-10, and others (11;12). This effect is partially blocked by anti IFN-γ mAb (11). The lack of systemic effects of IFN-γ (i.e. the lack of IFN-γ or MHC induction in the contralateral kidney) probably reflects the small quantity of IFN-γ produced during local injury. We showed MHC class I and II induction in the absence of IFN-γ. Immunohistology confirmed that the class I and II expression are induced in epithelial cells, and class II positive interstitial cell staining is also increased. The candidate stimuli

inducing local MHC expression in the GKO mice in response to injury include cytokines (IFN-α/β, TNF-α, and many others) and components of the extracellular matrix (43). Perhaps synergy between two or more signals is operating in the local injury response.

Renal injury induces increased expression of MHC genes and the antigen presenting machinery by mechanisms both dependent on and independent of IFN-γ/IFN-γRα (13;37), raising the question of the mechanisms. I presume that the IFN-γ/IFN-γRα-dependent component is mediated by natural killer cells, which are major sources of IFN-γ production and will be recruited to injured areas by the chemokine response. Antigen non-specific recruitment and activation of T cells may also play a role. In contrast, we suspect that the IFN-γ/IFN-γRα independent component of this response seen in gentamicin toxicity experiments (15;20) may represent an intrinsic response of the stressed and regenerating epithelium (i.e. to changes in integrin signals from extracellular matrix) but may also reflect non-IFN-γ cytokine production from inflammatory cells. In short, the mechanisms of injury-mediated inflammation and the immune response have yet to be well-defined (57). The ability of injury to induce renal MHC expression bears no obvious relationship to the nature of the inducing stimulus. For example it is not dependent on reperfusion injury as was first proposed when we and others observed this effect after renal ischemia (12;11;58). The injury-induced increases in antigen presentation could be a link between renal injury and both autoimmunity and transplant rejection (57;59).

Tissue injury increases the expression of CIITA mRNA *in vivo*. The changes in CIITA parallel those of class II mRNA and CIITA is regulated in part by the transcription factor IRF-1 and by non-IFN-γ mechanisms (15). The induction of CIITA was demonstratable in mice lacking IFN-γ, and also in mice lacking IRF-1, but at a reduced level. Hence, I suggested that CIITA mediates the increase in class II expression in tissue injury, and that the increase in CIITA mRNA expression reflects multiple influences, including IRF-1. Data from the CIITAKO mouse proved that CIITA does mediate class II induction in a complex injury response. Local tissue injury induces MHC molecule expression in the injured and/or recovering epithelial cells and an accumulation of a class II positive interstitial infiltrate. The injury-associated changes are not as large in magnitude as the systemic IFN-γ response to inflammation but are of great complexity reflecting changes in at least PI and PIV but also in PIII, and reflecting both population changes and transcriptional changes.

CIITA induction in tissue injury appears to be largely independent of IFN-γ, since all three CIITA promoter mRNAs and class II expression were induced in both in WT and in GKO mice after renal injury. We showed that basal class II expression is close to normal in GKO and IRF-1KO mice (9;13), suggesting that CIITA expression independent of IFN-γ and IRF-1plays a role in determining basal class II expression. By immunohistochemistry it is clear that CIITA is responsible for basal class II expression in kidney since the interstitial cells did not stain for class II in the CIITAKO mice using the M5 monoclonal antibody. Basal expression of class II in mouse organs such as heart and kidney

presumably reflects induction by local signals, which could include collagen (60), IFN-γ and other cytokines or growth factors, and other signals (61). Acute tissue injury increases local expression of class I and II, as well as many cytokines and growth factors (11;14;62;63). By immunostaining, the increase in class II expression in injured kidneys occurs in epithelium as well as interstitial cells in both WT and in GKO mice (13). The change in the epithelial cells occurs at day 3-7, and reflects class II gene induction in those cells by unknown mechanisms. The increased CIITA expression in renal injury probably reflects CIITA expression by epithelium, since the changes correlate with the tissue staining for class II. However, from these experiments I cannot determine whether there is a component of CIITA expression induced locally by IFN-γ. The localisation of CIITA protein should be established by immunostaining of tissue sections once monoclonal anti-CIITA antibodies are available.

Section conclusions.

It is most satisfying to have finally delineated a pathway through which injury-dependent mechanisms can operate, at least for class II (Figure 7.3). While class I and II are both only partially dependent on IFN-γ and IRF-1 for MHC induction, class II is completely dependent on CIITA even during the injury response. While the relevance of MHC expression to the rejection of a transplant remains unsolved, CIITA emerges as an attractive point at which to control immune response in tissues. The fact that people and mice with disrupted CIITA genes have no developmental defects in their non-lymphoid tissues indicates that

CIITA is a non-redundant essential step in the immune response, and thus could be a useful target for intervention. Interventions which were able to control CIITA expression or function could dampen immune response without adverse effects in other systems. The present studies suggest that such interventions could have the potential to reduce the consequences of non-specific injury, especially if applied as a gene therapy for the graft prior to transplant.

D. The Response To Transplantation

During an inflammatory response such as graft rejection, endothelial cells mediate the migration of immune cells from the circulation into the tissues. Endothelial cells express class I in the basal state and upregulate class II in response to IFN-γ. Several *in vitro* studies have shown that cultured endothelial cells can mediate proliferation of alloreactive T cells (64). However, the recognition of the endothelial cells implies direct recognition of allo-MHC by host T cells. But how do host T cells become primed to the graft in order to recognise donor antigen or MHC? Naïve T cells are not thought to traffic through tissue, perhaps due to their L-selectin (CD62L) expression. Only upon activation can T cells down regulate L-selectin and upregulate CD44 to facilitate entry to the tissues. The more likely route of priming is from autologous APC that have taken up apoptotic cells of the graft and then trafficked to the draining lymph nodes to stimulate T cells there. Those activated T cells can then travel to the site of inflammation and extravasate to the tissue. Using this paradigm of a primary

immune response, it seems unlikely that the direct pathway of recognition would activate the T cells.

The hypothesis is that IFN-γ is a destructive mediator of graft rejection because of its proinflammatory role in the immune response. However the role of IFN-γ in graft rejection is much more complex. Wild-type mice have increased class I and II molecule levels both in the graft and in the host during allograft rejection. Class I and II are upregulated on parenchymal and endothelial cells in the graft. Wild-type grafts into GKO hosts show very little MHC induction, yet GKO hosts reject allografts in an accelerated fashion with increased infarction and haemorrhage (65;66). GKO and wild-type mice show similar cytotoxic T cell gene expression patterns and circulating alloantibody levels. Nevertheless it is difficult to determine whether the protective effect of IFN-γ is on the host or on the graft. GRKO kidney allografts in normal mice cannot receive IFN-γ, and have increased vascular injury and infarction at day 7. Thus IFN-γ transiently protects grafts against vascular damage and infarction (39;67), by a direct effect on the graft. Whether protection against injury is due to the MHC induction or to other effects of IFN-γ (i.e. induction of endothelial protective genes) is not known.

The GRKO allografts have greatly reduced MHC class I and II induction in the graft, and had reduced infiltration by cells bearing CD3, CD8, and CD45 markers and a particularly marked reduction in CD4 cells. This observation suggests that donor MHC induction in the graft may promote infiltration by host CD4 T cells, and that MHC induction in the transplant is biologically significant for the T cell response. A hypothesis could be that IFN-γ actually protects against

alloantibody injury, but whether this is due to MHC induction or another action of IFN-γ is uncertain. Indeed the apparent paradox may be due to the multiplicity of potential effector mechanisms operating on the graft – alloantibody versus T cells. Another mechanism behind the reduced cell counts could be due to reduced blood flow resulting in increased ischemia. Thus IFN-γ (and potentially MHC induction) could protect against alloantibody or non-specific tissue destruction while promoting the somewhat slower T cell mediated rejection.

The role of class II in the graft is also complex. One would hypothesise that the absence of class II would be protective in a vascularised allograft. In our model, the grafts had no discernible advantage or disadvantage from the lack of class II in the graft. This was surprising since Ting's group has seen some protection in a heart allograft model (personal communication) in their CIITAKO mice. Results from the CIITAKO mice in transplant models have not been published, so it is difficult to know the precise details of the CIITAKO system. However, our results demonstrate that direct recognition of the graft by CD4 cells is not necessary for rejection. Thus the indirect pathway in the graft must be the major route of allograft rejection. Indeed, previous *in vitro* studies have also implicated the indirect class II pathway in the recognition of endothelial cells.

One could hypothesise that mechanisms dependent on class I may mediate the rejection response. However, one is still left with the problem of how the naïve cytotoxic T cell would become primed in order to become an effector T cell. This maturation is thought to require CD4 T cell help (68). Thus the

immune mechanisms of graft rejection are mediated through class II, through the indirect pathway, at least as far as the graft is concerned.

Section conclusions

Allograft rejection is a complex set of stimuli occurring at various stages in the life of the graft. Acute graft rejection is thought to be mediated by immune factors while chronic allograft dysfunction is likely mediated by other mechanisms such as stress factors. We have shown that an early rejection episode is not dependent on the expression of class II on the graft. Furthermore, IFN-γ mechanisms are protective early in the response, but may also be detrimental since IFN-γ induced MHC expression in the graft.

The congruence between the phenotypes of GKO and GRKO mice confirms the unique dependency of IFN-γ on IFN-γR and vice versa, and the unique role of the IFN-γ/IFN-γR unit in renal MHC regulation *in vivo*. Certain effects of IFN-γ on immune regulation as well as the host defence problems encountered by humans and mice with IFN-γ and IFN-γR deficiencies may reflect the defects in antigen presentation and MHC regulation, rather than to direct effects on lymphocytes. The role of the tubular epithelium in antigen presentation has been explored elsewhere (7;69-71) but remains unresolved. Regulation is not limited to class II, since class I and many other components of the antigen presenting system are also induced in kidney in response to allograft rejection and various other stimuli discussed here. It will thus be instructive to

explore how the high responsiveness of the kidney to stimuli, both through the IFN-γ/IFN-γR system and independent of it, impacts the propensity toward autoimmune diseases (72).

III. FUTURE DIRECTIONS

The experiments described herein define a role for inflammation and injury in the induction of antigen presentation. We now know many of the mediators of antigen presentation in a non-lymphoid tissue, which were unknown previous to these studies. What remains is to define the roles of these stimuli in the course of graft rejection. Experiments such as ischemically injuring a kidney and then transplanting it will bring the experiments closer to the human transplant condition. With the addition of a microsurgeon to the group, those experiments are now feasible.

These experiments would also benefit from an inducible knockin (on a CIITAKO background) for each promoter for CIITA; thus we could discern exactly the effect of stimulation through a specific promoter on class II expression in any given cell type.

The studies of CIITA promoter usage would benefit from an in situ PCR system and a set of CIITA promoter knockout mice. Using these technologies, the location of promoter use would be attainable, and the role of each promoter and cell set that expresses that promoter could be identified. The fine-tuning of the class II system makes it an obvious target for manipulations that would benefit specific disease states.

IV. CONCLUSIONS

1. Basal MHC class I in the kidney is dependent on IFN-γ
2. Autoinduction of IFN-γ does not play a role in MHC expression in the kidney of GKO mice
3. The kidney has a responsive antigen presenting machinery
4. CIITA in human and mouse is highly homologous
5. CIITA is required for all class II induction in the kidney
6. The absence of class II on a renal allograft is not protective
7. CIITA uses PIV in kidney in response to systemic stimuli
8. CIITA uses all promoters in response to renal injury

V. FIGURES

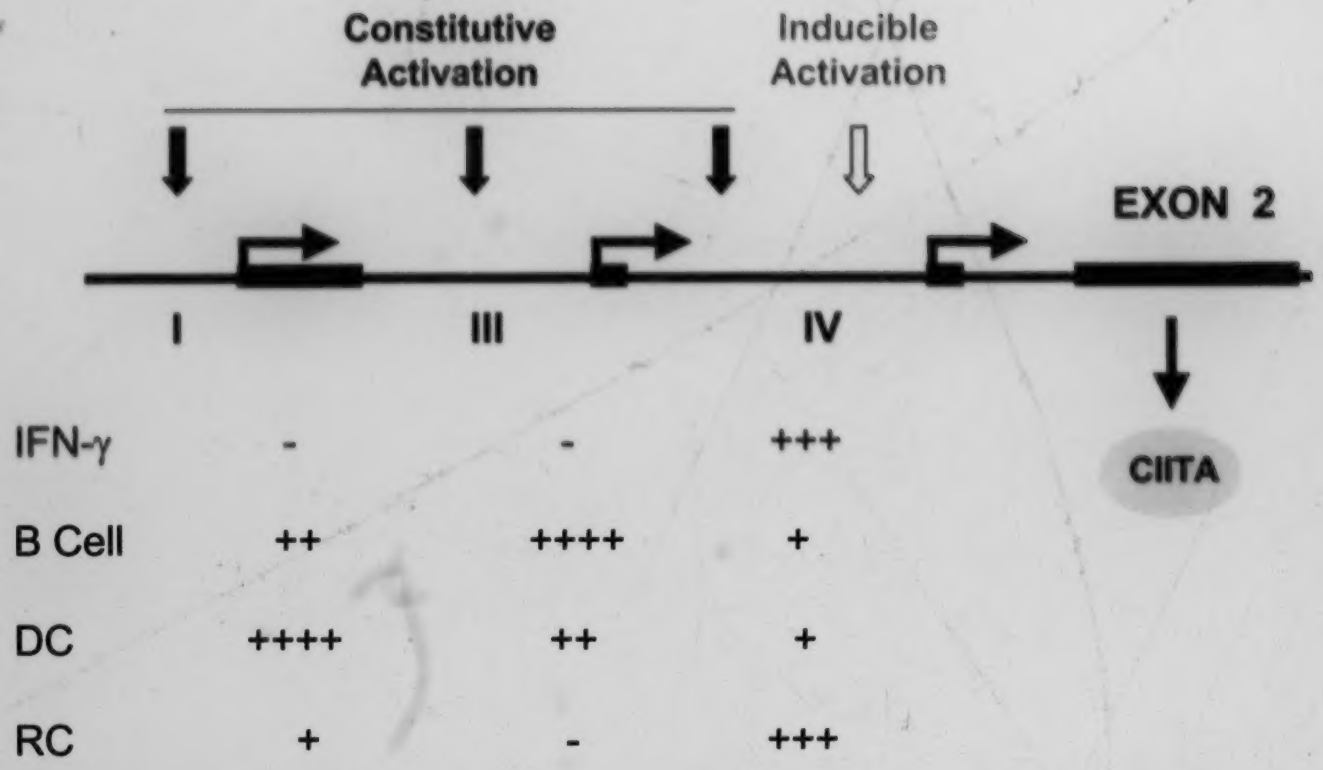

Figure 7.1 : Genomic arrangement of the mouse CIITA promoters and Exons 1. The predicted expression patterns of each promoter is also noted. Dendritic cell (DC), renal cells (RC). Renal cell populations are highly IFN-γ and PIV dependent, even in the basal state.

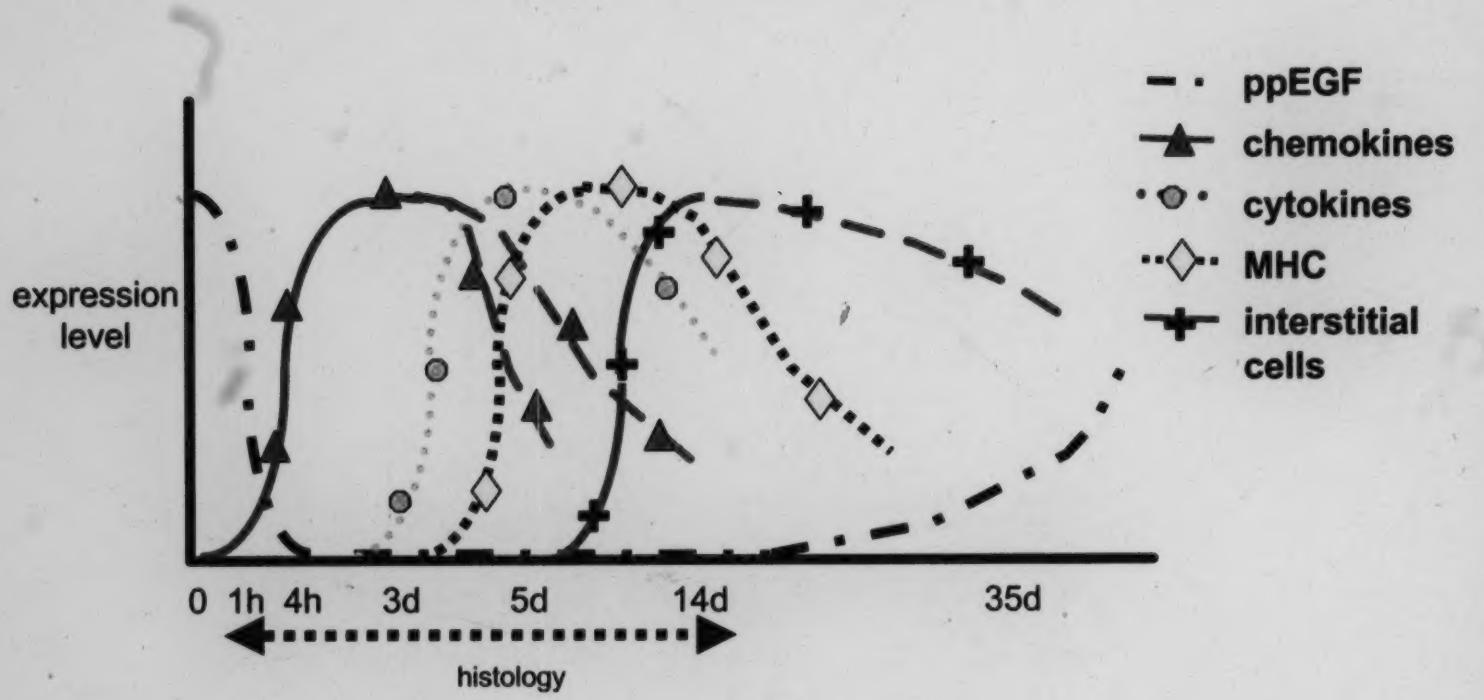

Figure 7. 2: Injury induces many changes in the kidney that are histologically normal by day 14. ppEGF, pre-pro epidermal growth factor; chemokines and cytokines determined by mRNA analysis; MHC and interstitial cell levels determined by histology.

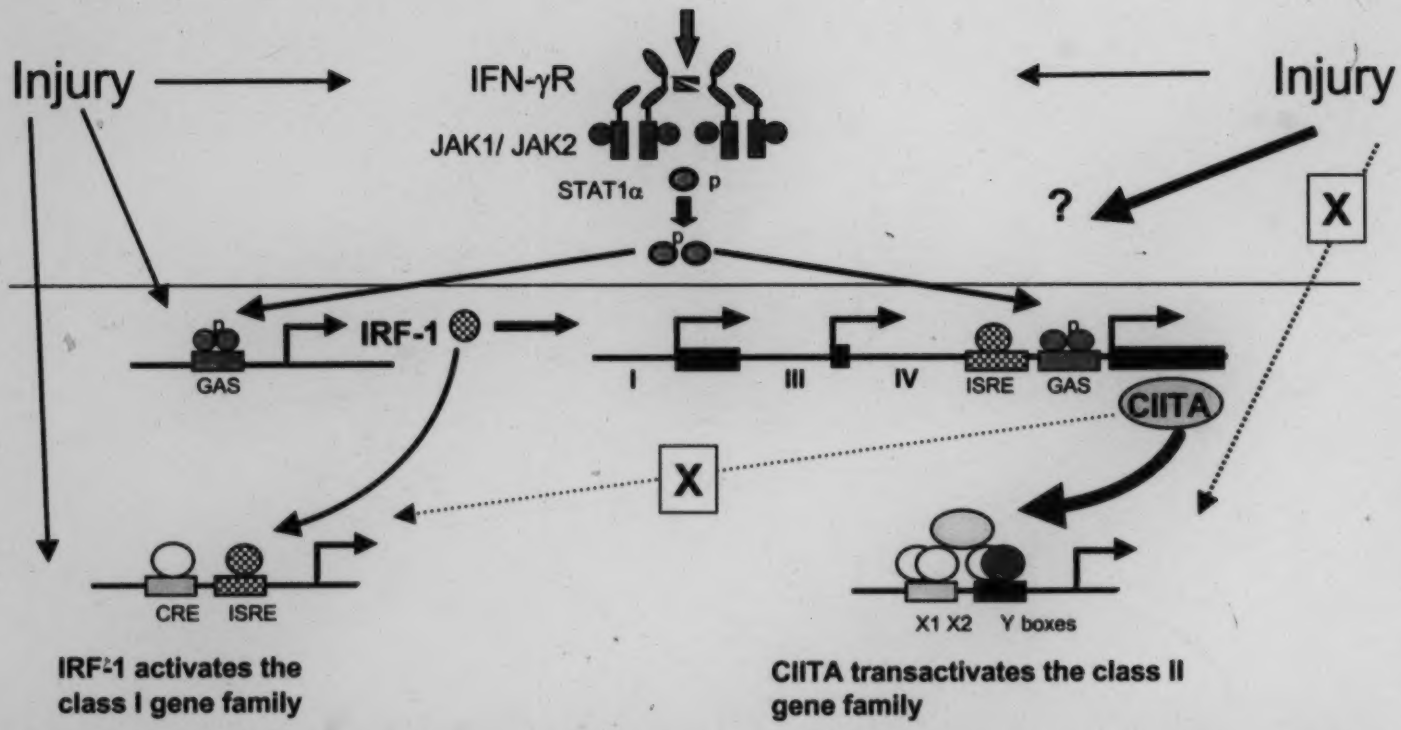

Figure 7.3 : IFN-γ induces antigen presentation through target genes. The targets themselves may be transcriptional activators such as IRF-1 and CIITA. Other transcription factors not shown bind each promoter . How injury influences MHC regulation is not known. Injury induces class I and II through IFN-γ as well as independent of IFN-γ. However, all injury and even transplant rejection induces class II via CIITA. We do not know how injury activates the various promoters of CIITA. CIITA does not activate class I in kidney.

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Education

1994-present University of Alberta, Edmonton, Canada. Department of Medical Microbiology and Immunology Ph.D. Candidate. Teaching Designation obtained.

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1989-1994 University of Calgary, Calgary, Canada.

B.Sc. Cellular, Molecular, Microbial Biology and Psychology (double major). May, 1994.

Honors and Awards

- Alberta Heritage Foundation for Medical Research Studentship 1996-present.
- University of Alberta Teaching Award, 2000
- Faculty of Medicine Teaching Award, 2000
- Graduate Students Association Service Award, 2000
- Canadian Society for Immunology and Serotec.

Student Poster Award. 1997.

- Faculty of Medicine 75th Anniversary Award, University of Alberta, 1995-1996.
- J. Gordon Kaplan Graduate Student Award, University of Alberta, 1995.
- University of Calgary Entrance Award, 1989.
- Alexander Rutherford Scholarship, University of Calgary, 1989.
- Royal Canadian Legion Bursary (Leadership and Academics), 1989.
- M. G. "Bud" Atkin Community Service Award, 1989.
- Alberta Bursary Program (in French) to CEGEP (Jonquiere, PQ), 1987.
- Rotary International Scholarship: Student Exchange to Brazil, 1987-1988.

Refereed Publications

1. Takei, Y, **Sims, T.N.**, Urmson, J., and Halloran P.F. The central role for IFN-γ receptor in the regulation of renal MHC expression. (2000) JASN. 11:250-261.
2. **Sims, T.N.**, Goes, N.B., Ramassar, V., Urmson, J., and Halloran, P.F. In Vivo class II transactivator expression in mouse is induced by a non-IFN-γ mechanism in response to local injury. (1997). Transplantation. 64(12):1657
3. **Sims, T.N.**, Elliott, J.F., Ramassar, V., Denney. Jr., D .W., and Halloran, P.F. Mouse CIITA: cDNA and amino acid comparison with human CIITA. (1997). Immunogenetics. 45:220-222
4. Halloran, P.F., Goes, N., Urmson, J., Ramassar, V., Hobart, M., **Sims, T.**, Lui, S.L., and Miller, L.W. MHC expression in organ transplants: lessons form the knockout mice. (1997). Transplant. Proc. 29:1041-1044

5. Goes, N., **Sims, T.**, Urmson, J., Vincent, D., Ramassar, V. and Halloran, P.F. Disturbed MHC regulation in the Interferon-γ knockout mouse: evidence for three states of MHC expression with distinct roles for IFN-γ. J. Immunol. 155: 4559-4566 (1995).

1. **Sims, T.N.**, Takei, Y, Urmson, J., Ramassar, V., and Halloran, P.F. Differential usage of the three class II transactivator (CIITA) promoters in normal and stimulated states in vivo. "Submitted"
2. **Sims, T.N.**, Urmson, J., Afrousian, M, and Halloran, P.F. The role of CIITA in class I and II regulation in vivo in response to inflammation, injury and transplantation. "In preparation"
3. **Sims, T.N.**, Pahl, A., Takei, Y. Ramassar, V. and Halloran, P.H. The in vivo regulation of the antigen presentation machinery in the kidney. "in preparation".

Invited Chapters

1. **T.N. Sims** and P.F. Halloran. MHC class II regulation in vivo in the mouse kidney. Microbes and Infection. 1(11):903-912. 1999.

Published Abstracts

1. **Sims T.N.**, Pahl, A., Ramassar, V., and Halloran, PF. Regulation of LMP7, TAP1 and H2-Ma levels in response to IFN-γ and injury in the kidney. FASEB

J. 12(4) part I: A590 (1998). Abstract accepted for poster presentation to the American Association of Immunologists.

2. Takei, Y., **Sims T.N.**, and Halloran P.F. Differential usage of the class II transactivator (CIITA) in vivo. FASEB J. 12(5) part II: A1064 (1998). Abstract accepted for oral presentation, American Association of Immunologists.
3. **Sims, T.N.**, Elliott J.F., and Halloran, P.F. CIITA in humans and mice: conservation of amino acid sequence and predicted structure. J. of Allergy and Clinical Immunology. 99(1,part 2): S10-11. (1997). Abstract accepted for poster presentation, American Association of Immunologists.
4. **Sims, T.N.**, Elliott, J.F., Parker, R., and Halloran, P.F. Cloning and sequencing of the mouse class II transctivator (CIITA) cDNA: comparison to human CIITA reveals high sequence conservation and potential structure-function relationships. (1997). Abstract accepted for oral presentation, American Society of Transplant Physicians.
5. **Sims, T.N.**, Vincent, D., Urmson, J., and Halloran, P.F. MHC regulation in non-lymphoid tissues of IFN-γ gene knockout (GKO) mice: evidence for a unique role for IFN-γ in MHC induction. FASEB J. 9(3): A491 (1995) . Abstract accepted for oral presentation, American Association of Immunologists.

TEACHING AND LEADERSHIP

Lectures and Presentations

- Western Conference in Immunology. July, 1999 Stanford, USA.
  Oral presentation
- American Society of Transplant Physicians. May, 1997. Chicago, USA.
  Oral Presentation
- American Association of Immunologists. April, 1995. Atlanta, USA.
  Mini Symposium

Teaching and Outreach

- Course Co-ordinator and Lecturer. Medical Laboratory Science 475, Clinical Immunology. September-December 1998, 1999 (video-taped Lectures)
- Continuing Medical Education. TIPS. April 1998
- Departmental Seminar: "Regulation of the antigen presentation machinery in response to inflammatory stimuli" April 3, 1998. (video-taped session)
- Department of Medical Microbiology and Immunology, University of Alberta. Immunology Teaching Assistant, 1996.
- University of Alberta Teaching Symposium. Participant, 1996-2000.
- AAI Advanced Course in Immunology. July 19-24, 1999
- WISEST Laboratory Session Instructor, March 1997, 1999
- Canada-Wide Science Fair. Senior Life Sciences Judge. May 1999.

- Edmonton Regional Science Fair. Judge for Junior High life sciences. March 1998, 1999.

Leadership

- University of Alberta, General Faculties Council. Graduate Student Representative, 1999-present
- Immunology Network Executive Committee. Graduate Student Representative, 1996-1998
- Immunology Network Retreat. Co-coordinator, 1997
- Faculty of Medicine Well-being Network. Student Representative, 1995-1997
- Department of Medical Microbiology and Immunology, Student Research Rounds.Founder and Organizer, 1995-1998.
- Graduate Student Association, Orientation Committee. Organizer and Volunteer, 1996
- Graduate Student Association, Department of Immunology, University of Alberta Student Representative, 1994-1995.
- Canadian Red Cross Society, Survivors of Torture Program. Founding Volunteer and Educator, 1990-1994.
- ROTEX Canada. Committee Member and Volunteer, 1989-present